I. THE EFFECT OF ULTRAVIOLET RADIATION AND OF SOFT X-RAYS ON THE SEDIMENTATION BEHAVIOUR AND LIGHT ABSORPTION OF PURIFIED HUMAN SERUM ALBUMIN

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Protein solutions are sensitive to ultraviolet light, and are coagulated by irradiation at the isoelectric point.[1] Coagulation can be prevented by irradiation either at reduced temperatures (0–4°) [Bovie, 1913] or in the presence of small amounts of acid or alkali (vide Arnow [1936] for references), but it then occurs if the temperature is raised, or the *p*H brought to the isoelectric point. It appears that coagulation of proteins by u.v. light takes place in at least two stages, a photochemical reaction independent of temperature, and an aggregation of the molecules attacked, a reaction of comparatively high temperature coefficient [Rajewsky, 1929; 1930; Clark, 1935]. The photochemical reaction causes increased light absorption [Spiegel-Adolf & Krumpel, 1927] and cleavage of the molecule, perhaps with the liberation of aromatic amino-acids [Mitchell, 1936].

Pedersen [1931], from preliminary experiments, reported that when serum albumin in solution at *p*H 3·5 was irradiated by u.v. light, the solution remained clear, the sedimentation constant doubled (indicating aggregation) and the diffusion constant showed a strong drift (indicating heterogeneity).

Previous investigations have not produced much information concerning the state of aggregation of the denatured protein molecules remaining in solution, either before or after the production of a precipitate, or concerning the effects of the substances causing the increased light absorption in irradiated protein solutions, or of *p*H upon the course of the reactions which follow the initial photochemical reaction. The ultracentrifugal method of analysis is suited to the exploration of a protein system after denaturation since both cleavage and aggregation of the protein molecules will be revealed on the sedimentation diagrams either as distortion of the curves or as separate maxima.

In view of the above, and of the importance of actinotherapy in modern curative medicine, an ultracentrifugal and light absorption study has been made of the effects of u.v. and soft X-ray irradiations on solutions of purified human serum albumin.

Experimental

Three series of experiments were made: one near the isoelectric point, at *p*H 5·4, where the irradiation, performed at 30°, caused ultimate coagulation of the protein, and two at *p*H 7·4 (normal blood *p*H), one at 30° and the other at 0°

[1] Arnow [1936] has reviewed the literature on the effects of irradiation on proteins and amino-acids.

where all the solutions remained clear. In each series, equal volumes of protein solution were irradiated in a rotating tube for various periods of time, and ultracentrifuged under identical conditions after as nearly as possible the same lapse of time. Experiment showed that rotation without irradiation did not cause surface denaturation. Some stock albumin solution was ultracentrifuged at the beginning and the end of each series to make certain that the solutions were stable.

Preparation of material. Fasting blood, sufficient for Series A and B, was drawn from a healthy subject into sterile centrifuge tubes and the resulting serum heated to 56° for 25 min. for preservative purposes. The heating of serum to 56° for as long as $2\frac{1}{2}$ hr. has been found by McFarlane [1935, 2] to have no detectable effect upon its sedimentation diagram. The globulins were separated by half-saturation with $(NH_4)_2SO_4$ according to the directions of Adair & Taylor [1935] and an unsuccessful attempt was made to repeat their preparation of crystalline human serum albumin. When only tiny globules were obtained, the albumin was purified instead by precipitation three times with $(NH_4)_2SO_4$ to saturation. It was dialysed for 30 hr. against distilled water, electrodialysed for 16 hr. and then stored in sterile bottles at 3°. Micro-Kjeldahl N analyses were made using the Parnas & Wagner [1921] distillation apparatus, and the N content converted into concentration of protein by the factor 6·25.

For Series A, 2 ml. stock solution were diluted immediately before irradiation with 0·7 ml. distilled water and 0·3 ml. 10 % NaCl solution, giving an albumin concentration of 0·81 g./100 ml.; sterile technique was followed as far as possible.[1] At the end of the series, a solution so prepared gave a *p*H of 5·44 (glass electrode).

For Series B, 20 ml. stock solution were diluted with 10 ml. of buffer solution containing 84·1 ml. *M*/5 Na_2HPO_4 and 15·9 ml. *M*/5 KH_2PO_4 per 100 ml.; the *p*H (glass electrode) was 7·39. 3 ml. samples were withdrawn for each irradiation experiment.

After the completion of these experiments, a paper by Tiselius [1937] appeared which showed that in serum, heated at 56° for even 10 min., the albumin boundary suffers a large reduction in electrophoretic mobility and migrates near the β globulin; this may indicate incipient denaturation. The albumin used in Series A and B had been prepared from serum heated at 56° for 25 min.; consequently the same change in mobility had probably taken place. Our results and the observations of McFarlane [1935, 2] indicated that this change did not affect the sedimentation diagram, but the possibility remained that the heated material might be more susceptible to irradiation, or might react differently.

For this reason a new stock solution of albumin (for Series C) was made from further blood from the same human; all operations were performed at 0–3°. The globulins were removed by 57 % saturation with $(NH_4)_2SO_4$, which was necessary to prevent precipitation during subsequent dialysis. The albumin was precipitated 3 times by saturation with $(NH_4)_2SO_4$, dialysed 4 days, electrodialysed for 50 hr. between 0 and 5° and diluted with phosphate buffer to the same protein and buffer concentrations as for Series B; the *p*H (glass electrode) was 7·40. This stock solution was frozen for storage to prevent bacterial action, Tiselius [1937] having reported that freezing does not affect the electrophoretic behaviour of serum proteins.

During the experiments in Series C, it was observed that to bring about thorough mixing of the protein-rich layer formed at the bottom of the flask on thawing, more vigorous agitation was necessary than the careful swirling which had purposely been employed to prevent foaming. A number of the samples withdrawn (by pipette, from the bottom of the flask) were more concentrated

[1] Spiegel-Adolf & Pollaczek [1929] found delayed coagulation in bacterially infected solutions.

than 0·81 % protein (see initial areas, Table I, which are proportional to concentrations of centrifugible protein). The concentration differences were not great enough to influence the sedimentation constant or spreading coefficient, which are independent of this factor at low concentration, but did affect all values which are concentration-dependent (curve area, height of sedimentation maximum, extinction coefficient). Analysis showed that the samples irradiated 1 and 2 hr. in Series C contained 0·814 and 0·819 % albumin respectively, so that for these comparison with Series A and B is permissible for all values. The solution used for determination of the sedimentation velocity of the untreated material was about 4 % too concentrated (see initial areas, Table I; no solution available for micro-Kjeldahl analysis). For the relationship shown in Fig. 2, the measured height of the sedimentation maximum for this solution was therefore multiplied by the ratio of the initial areas, 0·0046/0·0048.

Radiation. The actual conditions of irradiation differed somewhat in the three series of experiments, but remained constant during each series. Directly after irradiation, 0·5 ml. of the solution was removed for the immediate determination of its U.V. absorption spectrum; the remainder was placed in a small sterile bottle, stored at 3° overnight and ultracentrifuged the next morning.

Series A. A Uviarc, with a reflector but without a shield in front of the lamp, was the source of U.V. light. The solution was contained in a quartz tube (12·5 cm. long, outer diameter 1·3 cm., inner diameter 1·05 cm.) stoppered with a paraffined cork and placed parallel to the arc, with its axis horizontal, at a distance of 20 cm. The tube was rotated about its axis at *ca.* 80 r.p.m. to ensure uniform exposure of all parts of the solution, and cooled with an electric fan. Under these conditions the temperature rose immediately to 28°, then increased gradually to 33° during the first hour, and to 35° during the next three. The solutions were irradiated for 5, 10, 15, 30 and 55 min. respectively; 30 min. irradiation caused a slight opalescence, and 55 min. a definite cloudiness of too small a particle size to be retained by fine filter paper. Some of this solution was also irradiated under similar conditions in a pyrex tube of the same dimensions; when a slight opalescence appeared after 4¼ hr. the irradiation was stopped.

Series B. The same arrangements as in Series A were used, but to minimize the heating of the solution by the arc, and to reduce fluctuations in intensity of irradiation due to erratic cooling of the arc by the electric fan, the lamp was protected by a quartz window. The distance from the arc to the quartz tube was 30 cm. instead of 20 cm. as in Series A. A Photronic cell was placed beneath the rotating tube to detect changes in the amount of radiation falling on the tube.

The solutions in Series B were irradiated for 15 and 30 min., and 1, 2 and 3 hr. The average temperature varied between 27·5 and 31° from experiment to experiment although the maximum deviation during any one irradiation was 2°. The average value of the current given by the Photronic cell was constant from experiment to experiment to within ±2 %. Because of the greater distance of the arc from the solution, 2 hr. irradiation in this series corresponded very nearly to 55 min. irradiation in Series A, but because of the higher *p*H, the solutions remained clear even after 3 hr. irradiation.

A portion of the solution used in this series was exposed for 3 hr. to the continuous radiation from a soft X-ray tube (Cu target, 100,000 V.), the total dose used being 29,000 r.u. (measured with a Victoreen R-meter). In the absence of a detectable change in the albumin solution, no further irradiations with X-rays were made (see Table I).

Series C. To separate the effect of irradiation as far as possible from the effect of heat, the solutions in this series were kept below 1° during irradiation by ice-

water flowing over the rotating quartz tube. The irradiated solutions were stored in ice-water (not frozen, to prevent possible aggregation of the denatured protein) until placed in the centrifuge cell, and ultracentrifuged at the lowest convenient temperature (21°), in order to minimize possible aggregation during the run. (Clark [1935] found that coagulation of isoelectric egg albumin irradiated at 4° has a comparatively high temperature coefficient, but occurs only slowly below 20°.) During the 2 hr. irradiation the Photronic cell readings were constant at 70 to within ±2%; during the 1 hr. irradiation the average reading dropped to 62, constant to within ±3%. The lamp burned out before a third irradiation could be made. The radiation doses in this series are thus not proportional to exposure time; allowance has been made for this variation in the graphical presentation of the data (Fig. 2), on the assumption that the intensities of all wave-lengths were reduced in the same ratio. The uncertainty in this assumption does not seriously affect the conclusions drawn.

Light absorption. Absorption spectra were obtained using the Hilger echelon cells [Twyman *et al.* 1931–32; Twyman, 1933] with a medium-sized Bausch & Lomb quartz spectrograph, by technique previously described [McDonald, 1936]. The absorption spectra are shown in Fig. 1. The position of the absorption bands are correctly represented but, because of a systematic error in the apparatus [vide Magill *et al.* 1937, Fig. 12], the ordinates do not show the absolute extinction coefficients, but give values which are too high by a factor which varies somewhat from wave-length to wave-length. At wave-lengths corresponding to the absorption maxima the measured values were found to be too large by a factor of 1·1. The corrected heights of the absorption maxima are plotted against the amount of irradiation in Fig. 2 (I, II and III). Using the above correction factor, a value of 6·0 was obtained for the specific extinction coefficient of serum albumin. Svedberg & Sjögren [1930] found a value of *ca.* 5 over the *p*H range 5–8, and Smith [1929] a value of 5·8 at *p*H 7.

Sedimentation velocity. Measurements of sedimentation velocity were made in a standard Svedberg oil-turbine ultracentrifuge at a speed of 56,000–58,000 r.p.m., corresponding to a centrifugal force of about 215,000 times gravity at the middle of the cell. The temperature of centrifuging was 24–26° for Series A and B, and 21° for Series C. Sedimentation was followed by the refractive index method of Lamm [1928; 1929] using a cell 3 mm. in thickness and a scale distance of 10 cm. Photographs were taken every 10 min., and the runs were continued for approximately 2 hr. Solvent runs were made from time to time under identical centrifuging conditions [cf. Pedersen, 1936] to provide standards from which the displacements caused by sedimentation of the protein were measured. These displacements were plotted against the distance from the centre of rotation to give the usual sedimentation diagrams.

All experimental conditions were kept as constant as possible from run to run, so that in any series the relative heights of sedimentation curves representing equal times of centrifuging and of diffusion give, to a first approximation, the relative concentrations of albumin of unchanged sedimentation constant in the irradiated solutions. The principal factor reducing the exactness of this relationship is the presence of aggregates incompletely centrifuged from the plane of the boundary, which increase the displacement. This has been minimized by selecting for the comparison a time of centrifuging (50 min.) sufficiently far advanced to ensure fair separation of the heavy particles, but early enough to avoid complications due to reflexion of particles from the bottom of the cell. The relative heights, using 100% for the untreated solution, are given in Fig. 2 (IV and V).

The sedimentation constants were calculated by the usual equation [Svedberg, 1933; 1937]:

$$s_{20} = dx/dt \,.\, 1/\omega^2 x_m \,.\, \eta_T/\eta_{20} \,.\, (1 - V\rho_{20})/(1 - V\rho_T)$$

and are listed in Table I. The sedimentation constant, $s = 4{\cdot}5 - 4{\cdot}6$, for the untreated material in Series A and for all but two determinations of Series C, is in good agreement with the published value, $s = 4{\cdot}5$ [Svedberg, 1937]. For Series B, and two determinations in Series C, the values are lower. This is discussed later.

Representative sedimentation diagrams are shown in Figs. 3 and 4. To avoid repetition, the diagrams for untreated albumin in Series B and C, and for X-ray-treated albumin in Series B, have been omitted; they were similar in appearance and symmetry to that of the untreated solution in Series A.

To estimate the relative amounts of centrifugible material in the various solutions [vide McFarlane, 1935, 1] the areas enclosed by the sedimentation curves were converted into absolute units, and, to correct for the dilution caused by centrifuging in a sector-shaped cell in a field of varying intensity [Svedberg & Rinde, 1924], multiplied by the factor $(x_t/x_0)^2$. Since the refractive indices (measured, at 26°, with an Abbé refractometer using a sodium lamp as light source) of the irradiated serum albumin solutions were identical with those of the unirradiated solutions, the corrected areas so obtained are proportional to the concentrations of centrifugible protein. The initial and final areas for each experiment are listed in Table I.

Spreading coefficients were calculated [Lamm, 1929] from the half-width, u, of the sedimentation curves at a height, h, where

$$h = \text{maximum height}/\sqrt{e} = 0{\cdot}606 \text{ maximum height.}$$

The values of u^2 were plotted against the time of centrifuging; the spreading coefficients, or "apparent diffusion constants", were given by the slopes of these curves [Loughborough & Stamm, 1936]. Constant values were obtained for the untreated solutions and for those which showed no marked change; these are listed in Table I. The others showed a drift with time, and single values can therefore not be given. In the solutions irradiated for the longest periods of time (A7, A8, B6, B7, B8, C3) there were discontinuities in the u^2-time curves; these represent the separation of discrete boundaries composed of particles whose concentration is sufficient to cause pronounced widening of the curve at the height considered.

The spreading coefficients calculated from the curve areas [McFarlane, 1935, 1] are listed in Table I. For characterizing mixtures this method of calculation is preferable to the one given above because it includes the spreading due to all particles present, and not only to those of sufficient concentration to widen the curve appreciably at the height considered. Further, for the untreated solutions, the method gave values which are in better agreement with the published values of the diffusion constant, 6·17 [Svedberg, 1937]. For a heterogeneous mixture the spreading coefficient has no physical meaning, but does provide an index of the range and relative concentrations of particle sizes.

Discussion

Absorption spectra. The absorption spectra (Fig. 1) show that at both pH the light absorption increases regularly as irradiation proceeds; the increase is general for wave-lengths greater than 2400 Å. but most pronounced at the characteristic protein maximum. For the same radiation dose (Fig. 2), the

increase in light absorption is greater at *p*H 5·4 than at *p*H 7·4. This suggests that the reactions causing the increase are inhibited at the higher *p*H.

As a rule the irradiation of amino-acids, dipeptides and related compounds containing only aliphatic radicals causes increased absorption of wave-lengths less than 2500 Å., while irradiation of those containing aromatic nuclei causes increased absorption at wave-lengths greater than 2400 Å. [Allen *et al.* 1937].

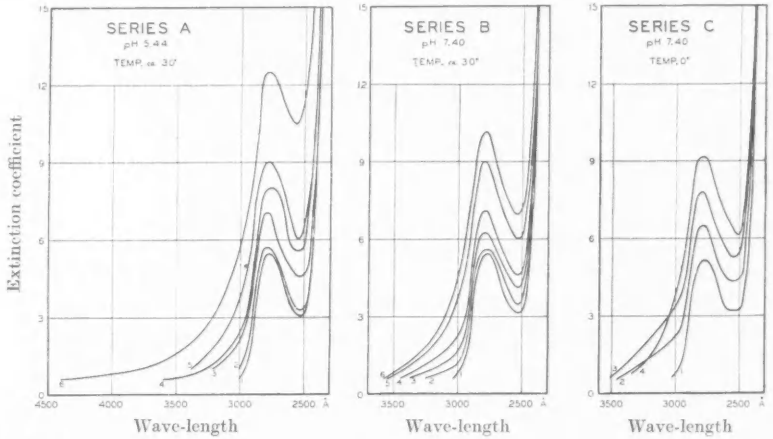

Fig. 1. Increase in absorption spectra with irradiation. Irradiation: Series A, 1, untreated; 2, 10 min.; 3, 15 min.; 4, 4·25 hr. in glass; 5, 30 min.; 6, 55 min. Series B, 1, untreated; 2, 15 min.; 3, 30 min.; 4, 1 hr.; 5, 2 hr.; 6, 3 hr. Series C, 1, untreated; 2, 1 hr.; 3, 2 hr.; 4, 2 months after 2 hr. irradiation.

Too little is known about the absorption spectra of individual amino-acids and polypeptides, and of their photolytic products, to ascertain the specific reactions responsible for the increased absorption of irradiated proteins. In general, U.V. light and cathode rays convert primary and secondary amino-acids into aldehydes, and tertiary amino-acids into ketones, by the addition of oxygen and water and the elimination of ammonia, carbon dioxide and water [Allen *et al.* 1937; Neuberg, 1908; 1910; Harris, 1926; Rao & Dhar, 1934; Becker & Szendrö, 1931]. The rate of ammonia cleavage varies from compound to compound, and is more rapid in an acid than in an alkaline medium [Lieben & Urban, 1931]. Peptones and proteins also yield aldehydes, ammonia and carbon dioxide, a fact which can be explained only by assuming that at some stage of the process hydrolysis of the peptide linkage takes place. Mitchell [1936], from a study of the effect of U.V. light on monolayers of egg albumin, has suggested that the primary reaction is the hydrolysis of CONH linkages adjacent to aromatic residues, with liberation of tyrosine, tryptophan, phenylalanine and perhaps histidine, since these groups are responsible for the high light absorption of the protein in the range 2500–2900 Å.

The concentrations of centrifugible material in the irradiated solutions also point to a reaction of this character. Table I gives the areas enclosed by the sedimentation curves, to which the concentrations of centrifugible material are proportional. All solutions which remained clear, and from which, therefore, none of the albumin had been precipitated, gave *initial* areas that were approxi-

mately the same as for unirradiated albumin. This shows that the non-centrifugible material split off by the action of the light constituted only a few % of the total weight. Tyrosine and tryptophan comprise respectively 4·66 and 0·53 % of the weight of serum albumin [Folin & Marenzi, 1929], phenylalanine 2·3 % [Stenström & Rheinhard, 1925], histidine 1·95 % [Block, 1934]; together they amount to about 9·4 %. The sedimentation data (Fig. 2, V) show that 3 hr. irradiation at *p*H 7·4 caused modification (with respect to sedimentation constant) of about 70 % of the serum albumin. Complete hydrolysis of all aromatic amino-acids in this modified fraction of the albumin would represent, therefore, a loss of about 6·6 % as non-centrifugible material. The observed loss, calculated from decrease in initial area (Table I), is not more than 4·5 % and is probably less, since part of the drop in initial area may be due to the removal of a small concentration of heavy aggregates. The diminution in area is thus consistent with the view that only aromatic amino-acids are hydrolysed from the molecule.

However, this reaction cannot be responsible for the increased light absorption. Tyrosine, tryptophan and phenylalanine have molecular extinction coefficients of 1576, 5900 and 180 respectively [Gróh & Hánák, 1930]. Calculation, using the above figures, shows that a solution containing as free acids the same concentrations of these amino-acids as are present in a 1 % solution of serum albumin would have an extinction coefficient of 5·83, while the specific extinction coefficient of serum albumin is between 5·0 and 6·0 [Svedberg & Sjögren, 1930; Smith, 1929].

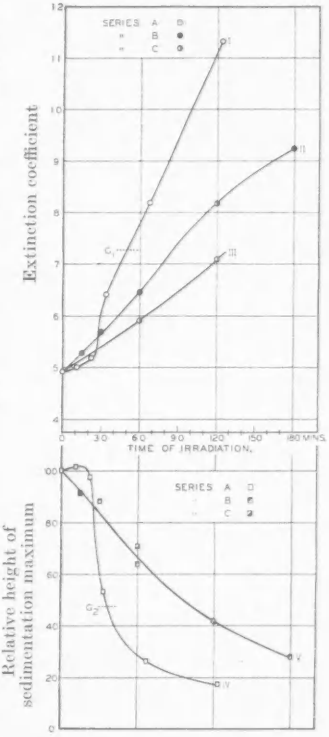

Fig. 2. Correlation between extinction coefficient and relative height of sedimentation maximum. The abscissae represent actual times of irradiation for Series B and C. The times for Series A are the actual irradiation times × 9/4, to compensate for the change in distance of the light source. G_1 and G_2 give the extinction coefficient and relative sedimentation height, respectively, of the solution irradiated 4·25 hr. in glass.

Arnow [1935] has shown that irradiation of egg albumin with α-particles causes increased absorption in isoelectric or acid solutions, decreased absorption in solutions alkaline to the isoelectric point. He has suggested that the increase may result from the photo-oxidation of phenylalanine to tyrosine and of tyrosine to dihydroxyphenylalanine ("dopa") which has a molecular extinction coefficient of 2500 [Abderhalden & Rossner, 1928]. Recently he has demonstrated that low concentrations of "dopa" are formed by the action of U.V. light on tyrosine [Arnow, 1937]. However, the conversion into "dopa" of all the tyrosine and phenylalanine present in serum albumin would cause only a 95 % increase in the extinction coefficient. This mechanism, therefore, even with the improbable assumption of complete conversion, is inadequate, by itself, to account for the increases in light absorption observed in the present investigation.

Table I

Treatment	Appearance	$s_{20} \times 10^{13}$	Spreading coefficient: Area	Spreading coefficient: Width	Curve areas: Initial	Curve areas: Final
					sq. cm.	
Series A. pH = 5·44; temp. approx. 30°						
1. Untreated (before)	Clear	4·67	6·53	5·4	0·0047*	
2. Untreated (after)	Clear	4·50	6·15	5·3	0·0047*	
3. Glass 4·25 hr.	Opalescent	4·84	12·8	—	0·0034	0·0034
4. Quartz 5 min.	Clear	4·49	5·59	5·4	0·0047	0·0047
5. Quartz 10 min.	Clear	4·61	7·19	5·8	0·0047	0·0047
6. Quartz 15 min.	Clear	4·89	16·8	—	0·0046	0·0037
7. Quartz 30 min.	Opalescent	4·98	24·8	—	0·0026	0·0020
8. Quartz 55 min.	Cloudy	4·92	18·7	—	0·0016	0·0013
Series B. pH = 7·40; temp. approx. 30°						
1. Untreated (before)	Clear	4·41	5·33	4·0	0·0046*	
2. Untreated (after)	Clear	4·47	5·25	4·0	0·0046*	
3. Quartz 15 min.	Clear	4·41	7·08	—	0·0046	0·0044†
4. Quartz 30 min.	Clear	4·29	7·44	—	0·0046	0·0043†
5. Quartz 1 hr.	Clear	4·44	16·9	—	0·0046	0·0039‡
6. Quartz 2 hr.	Clear	4·41	48·5	—	0·0046	0·0036‡
7. Quartz 3 hr.	Clear	4·41	73·0	—	0·0044	0·0031§
8. Quartz 3 hr. (repeated 6 days later)	Clear	4·33	80·0	—	0·0044	0·0035§
9. X-rays, 29,000 r.u.	Clear	4·39	6·24	3·7	0·0046*	
Series C. pH = 7·40; temp. 0°						
1. Untreated (before)‖	Clear	4·63	6·61	3·5	0·0048	
2. Quartz 1 hr.	Clear	4·57	17·1	—	0·0046	0·0040‡
3. Quartz 2 hr.	Clear	4·57	54·6	—	0·0046	0·0035‡
4. Heated, and rotated for 3 hr.‖	Clear	4·22	4·69	—	0·0071*	
5. Untreated (temp. of centrifuging, 25–26°)‖	Clear	4·23	4·72	—	0·0075*	
6. Untreated (temp. of centrifuging, 20–22°)‖	Clear	4·53	6·02	—	0·0076*	

* Average values over entire time of centrifuging: † 72 min., ‡ 60 min. and § 50 min. from start of centrifuging. ‖ Albumin concentration greater than 0·81%.

Immediately after irradiation, the solutions irradiated at 0° (Series C) showed less increase in absorption at the protein maximum, but more increase on the long-wave side of the protein band, than the solutions of the same pH irradiated for the same periods of time at 30° (Series B) (compare B4 and C2, B5 and C3, Fig. 1). Prolonged standing at 3° led to the disappearance of this difference, resulting in increased absorption below 3000 Å. and decreased absorption above 3000 Å. in both series of solutions, but to a far more limited extent in Series B than in Series C (compare B5, C3 and C4, Fig. 1). The effect of the temperature of irradiation was in this way eliminated, and identical spectra were obtained for the two series, a finding which is consistent with the observations of Rajewsky [1929; 1930] and Clark [1935] that the temperature coefficient of the primary photochemical process is unity. Since no changes could be detected in the absorption spectra of the unirradiated solutions which had been stored at 3° for the same lengths of time, it seems possible that part of the absorption at wave-lengths longer than 3000 Å., observed in the solutions irradiated at 0°, represents an unstable intermediate stage [Mirsky & Pauling, 1936, p. 444] which produces, on warming or on prolonged standing in the cold, the compound(s) responsible for the increased absorption at the protein maximum.

The two solutions irradiated 2 hr. at pH 7·4 were brought to pH 5·4 by dialysis at 3° through cellophane membranes against large volumes of $M/15$ phos-

phate buffer. The absorption spectra were taken first with the chilled solution, then again after warming to 30° for 20 min. Both solutions remained clear. Each gave the same spectrum before and after warming, but the two differed a little from each other, perhaps because of slight dilution during dialysis. The increased absorption, above that of the native protein, was in both instances reduced to about half what it had been before dialysis, i.e. to an extinction coefficient of 7. This suggests that while part of the increase results from cleavage products small enough to diffuse through cellophane membranes, much of it must arise either from reactions in the protein molecule without cleavage, or from cleavage products too large to diffuse through cellophane.

The solution of *p*H 7·4 irradiated 1 hr. at 30° was brought to *p*H 5·4 by the addition at 0° of *N* HCl; this caused a 4% increase in volume. Within the limits of error of the method, the absorption spectra measured before and after warming were identical with each other and with that of the solution before dialysis. Since the solution which was given approximately the same radiation dosage at *p*H 5·4 (Series A, 30 min.) gave much greater absorption, it appears that the action of the light is inhibited at *p*H 7·4.

Sedimentation velocity measurements. The most obvious result of irradiation is the progressive reduction in the heights of the sedimentation curves (Figs. 3 and 4); but in each series the sedimentation constants remain the same as for the native protein (Table I), except for a slight increase at *p*H 5·4 (Series A). These facts show that in the irradiated solutions the boundaries represent albumin of unchanged sedimentation constant, and that the concentration of such albumin is diminished by successively longer periods of irradiation. Irradiation also causes an asymmetrical broadening of the sedimentation curves to the right of the maximum, which demonstrates the presence of particles heavier than native albumin, and suggests that the protein molecules which have been attacked by the light coalesce to form aggregates. Resolution of the faster-moving boundaries was not sufficient for calculation of the sedimentation constants, but qualitative information concerning aggregate sizes can be gained from the curve areas and curve spreading.

For solutions irradiated longest, the curve areas (Table I) diminish as centrifuging proceeds, because the heavy aggregates, in order of size, have been centrifuged to the bottom of the cell and removed from the field of observation. At *p*H 7·4 the rate of this reduction increases with increase in irradiation time; this shows that as irradiation proceeds not only the concentrations but also the average sizes of the aggregates become greater. At *p*H 5·4 the separation of a visible precipitate causes a reduction in *initial* area, the greater the longer the effective period of irradiation; the further reduction in area during centrifuging shows that the heavy aggregates characteristic of the solutions irradiated 15 and 30 min. are present in lower absolute concentration in the solution irradiated 55 min., and are almost entirely absent from the solution irradiated in pyrex glass for $4\frac{1}{4}$ hr. The spreading coefficients show the same trend, a continuous increase at *p*H 7·4, and at *p*H 5·4 an increase to a maximum near 30 min. irradiation; but the greater magnitude of the variations emphasizes the differences in average particle size. These differences were also clearly shown by the variation of u^2, the square of the half-width of the curve at 0·606 times the maximum height, with time of centrifuging: at *p*H 7·4, each solution irradiated for 30 min. or more gave curves which indicated progressively diminished spreading, the result of the removal of the heaviest aggregates as the centrifuging proceeded, while at *p*H 5·4 the solutions irradiated for 30 and 55 min. both gave curves which indicated the gradual separation of particles sedimenting with velocities of the

same order of magnitude, and the absence of appreciable concentrations of heavy particles. The discontinuities in the u^2-time curves for the solutions irradiated longest (A7, A8, B6, B7, B8, C3) represent the separation of discrete boundaries each composed of particles whose concentration is sufficient to cause marked

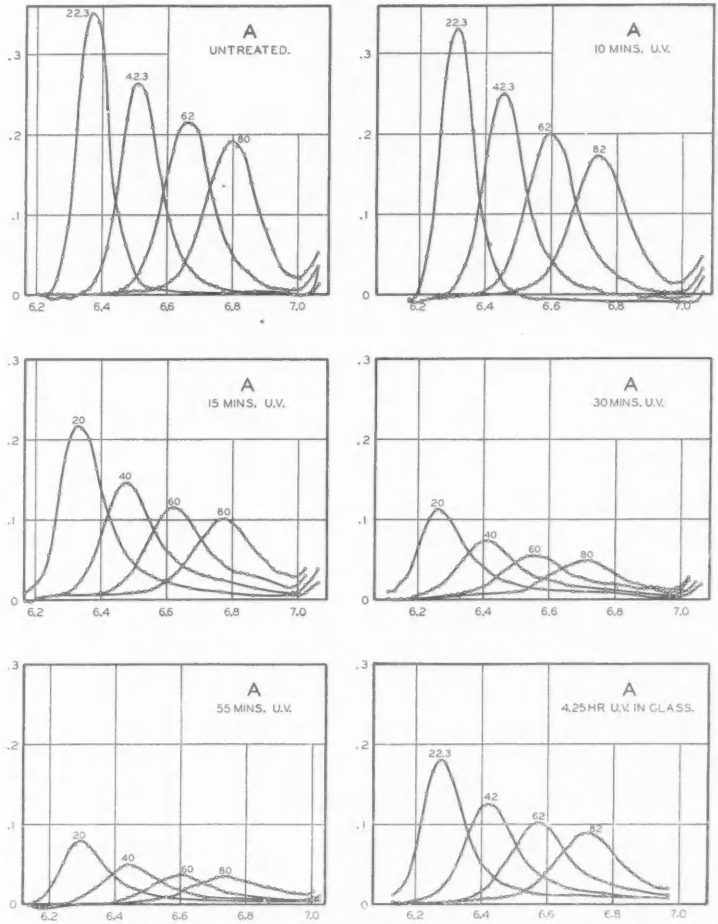

Fig. 3. Sedimentation diagrams. Series A. *p*H 5·44. Abscissae, distance from centre of rotation (X in cm.); ordinates, scale-line displacement (Z in mm.). The figures near the curve maxima represent the time in minutes from the application of the final driving pressure to the rotor. The figure under the series letter shows the duration of U.V. irradiation. Temperature of irradiation *ca.* 30°.

broadening of the curve at the height considered. The approximate sedimentation constants for these boundaries, estimated from the discontinuities, fell into two groups, one about 6, the other a little more than 7, and probably correspond to aggregates composed of two or three albumin residues.

The results as a whole indicate that in solutions sufficiently alkaline to prevent precipitation of the denatured protein, irradiation leads to the formation of increasing amounts of aggregated product of successively greater particle size,

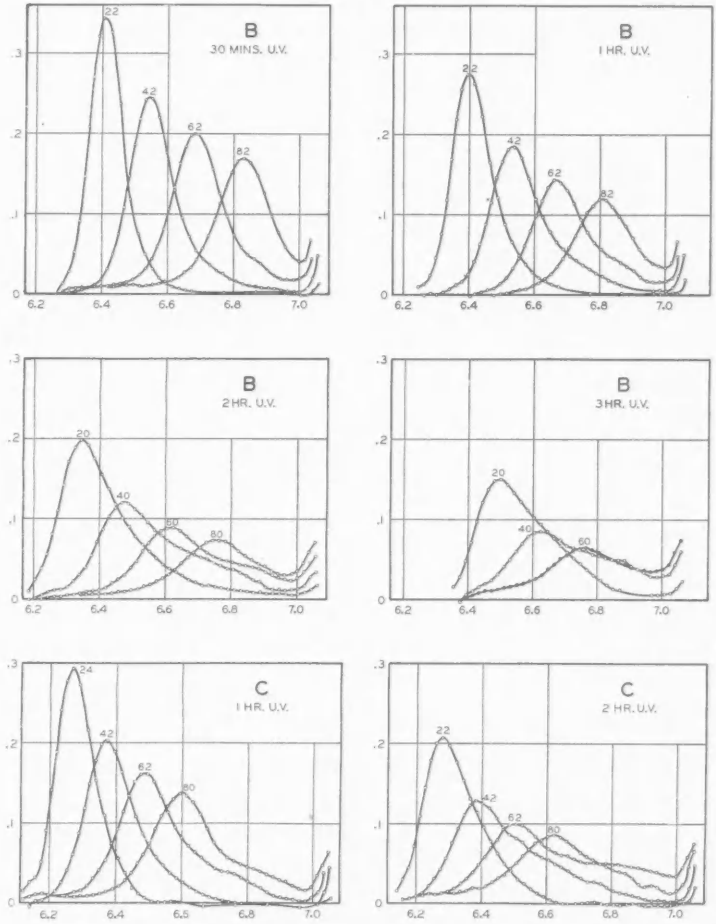

Fig. 4. Sedimentation diagrams. Series B and C. *p*H 7·40. Abscissae, distance from centre of rotation (*X* in cm.); ordinates, scale-line displacement (*Z* in mm.). The figures near the curve maxima represent the time in minutes from the application of the final driving pressure to the rotor. The figure under the series letter shows the duration of U.V. irradiation. Temperature of irradiation: Series B, *ca.* 30°; Series C, 0°.

but there appears to be a limit to the size developed. This type of aggregation seems to take place even at low temperatures, or else very rapidly at about 20°, because the precaution, observed in Series C, of irradiating and storing at 0° until the beginning of the ultracentrifuge run, had negligible effect on the aggre-

gation; this can be seen from the similarity of the sedimentation curves (Fig. 4), of the relative heights of the sedimentation maxima (Fig. 2, V) and of the spreading coefficients (Table I), to those of the solutions irradiated for the same times at 30°.

On the other hand, in solutions nearer to the isoelectric point there is no limit to the particle size, and a visible precipitate is formed at the expense of aggregates of intermediate size. Here the comparatively high temperature of irradiation (30°) has definitely influenced the particle size distribution. Because the coagulation process has a rather large temperature coefficient [Rajewsky, 1929; 1930; Clark, 1935], the solutions irradiated the longest were given the most favourable conditions for coagulation, and became most impoverished with respect to soluble aggregates, especially those of large size. The maximum in the spreading coefficient after about 30 min. irradiation in quartz, therefore, probably represents the stage of aggregation which immediately precedes the formation of a visible precipitate, first seen after 30 min. irradiation.

The absence of heavy soluble aggregates from the solution irradiated for $4\frac{1}{4}$ hr. in pyrex glass is undoubtedly the result of prolonged high temperature. The absorption spectrum of this solution is in all respects similar to those of the solutions irradiated in quartz. Since, as can be seen from Fig. 2 (I and IV), the heights of the sedimentation and absorption maxima correspond to about the same degree of change as that to be expected from approximately 20 min. irradiation in quartz, it seems reasonable to suppose that the reaction is the same in both quartz and pyrex, but that the difference in extent results from the greater transparency of quartz to the effective wave-lengths [Rajewsky, 1930].

The greater uniformity of the sedimentation constants for the observed boundary in solutions irradiated at *p*H 7·4 can probably be attributed to the preponderance among the aggregates of heavy particles which quickly separate from the main boundary; the upward trend at *p*H 5·4, to the higher relative concentrations of small aggregates, which, being incompletely resolved from the main boundary, cause an apparent increase in the sedimentation constant.

For equal irradiation at 30°, the reduction in the concentration of albumin of unchanged sedimentation constant is greater at *p*H 5·4 than at *p*H 7·4; this is shown in Fig. 2 (IV and V). It appears, therefore, that the aggregation process is inhibited at the higher *p*H. Gross coagulation, which is also inhibited at *p*H removed from the isoelectric point, is known to be a process distinct from the light reaction [Rajewsky, 1929; 1930; Clark, 1935]. Doubtless, therefore, even the simplest stages of aggregation must also be considered separate from it, though it is scarcely justifiable to conclude on this basis alone that the initial photochemical process, which may be considered to render the molecules susceptible to aggregation, is also inhibited at the higher *p*H. However, the correlation between reduction in albumin concentration and increase of light absorption, shown in Fig. 2, is fairly convincing evidence that the two are manifestations of the same fundamental change. It has already been shown that the reaction causing increase of light absorption is inhibited at *p*H 7·4; it thus seems likely that the photochemical reaction causing aggregation may also be repressed. This conclusion is supported by the fact that while solutions irradiated at *p*H 5·4 for 30 and 55 min. respectively showed opalescence and turbidity, the solutions which were adjusted to *p*H 5·4 *after* receiving equivalent amounts of radiation (1 and 2 hr.) at *p*H 7·4 all remained clear.

Young [1922], from measurements of the amounts of coagulum formed at the isoelectric point after irradiation at various *p*H, reached the opposite conclusion, that the reaction was progressively more rapid on both sides of the isoelectric

point. However, his solutions were not rotated during irradiation, and since the increase of light absorption has been shown to be less at *p*H 7·4 than at *p*H 5·4, a possible reason for his results may be the lower screening effect, at *p*H removed from the isoelectric point, of the irradiated layers nearest the light source.

Because of the uncertainty of the protein concentrations and radiation doses in Series C, a detailed quantitative comparison cannot be made with the results of Series A and B. However, the relative heights of the sedimentation maxima are, as far as can be ascertained, the same as for the solutions of the same *p*H irradiated at 30° (Fig. 2, V), while the maximum extinction coefficients are definitely lower (Fig. 2, III). The correlation between rise of absorption maximum and drop in sedimentation maximum is thus not apparent for Series C, the reason probably being that the absorption spectra, taken within 5 min. of the removal of the solution from the ice-bath, represent quite accurately the unheated solutions, while the ultracentrifuge data refer to the solutions warmed to 20–22° for about an hour during the loading and accelerating of the ultracentrifuge. After prolonged standing, during which the temperature had at one time risen to approximately 20° for about an hour, the solution irradiated 2 hr. gave an absorption spectrum identical with that of the solution irradiated 2 hr. at 30°. It appears that solutions of *p*H 7·4 irradiated at 0° tend very readily to assume the same state, with respect to both aggregation and light absorption, as those irradiated at 30°. It would thus seem, too, that the preliminary heating to 56° of the albumin solutions in Series A and B did not affect the reactions caused by the u.v. light.

Pedersen [1931] found that an irradiated solution of serum albumin of *p*H 3·5 gave a sedimentation constant about twice that of native albumin, presumably about 9. The apparent discrepancy with the present results can most probably be explained either by the difference in *p*H or by the greater boundary-resolving power of the refractive index method as compared with the light absorption method used by Pedersen, especially in the presence of non-centrifugible material of high light absorption. It is possible that in his experiments the unaltered albumin did not appear as a separate boundary, but was completely merged with the aggregation products, so that the measured sedimentation constant represented an average for the mixture as a whole.

The characteristic odour which has been mentioned by several investigators [Pedersen, 1931; Stedman & Mendel, 1926] was absent from our solutions. We are inclined to attribute this to the precaution of rotating the irradiation tube, which ensured more equal exposure of all parts of the solution, and prevented the accumulation of degradation products of high light absorption in the layers nearest to the source of illumination.

To determine whether rotation of the albumin solution caused any surface denaturation contributing to the observed results, especially in the solutions which had been heated to 56°, and which might, therefore, be more susceptible to denaturation, some of the solution used for Series C was heated to 56° for 25 min., rotated without irradiation for 3 hr. at 26–29° and ultracentrifuged at 25°. The sedimentation curves were similar in appearance and symmetry to those given by the untreated albumin, and showed no evidence of the aggregation which might be expected to result from denaturation. There was a drop in the sedimentation constant, beyond the limits of experimental error, from 4·6 to 4·2, and a drop in spreading coefficient, *D*, from 6·6 to 4·7 (see Table I), but further experiments showed that heating was the cause, not rotation. A sample of the untreated solution ultracentrifuged at a cell temperature of 25–26° gave the same

low constants, $s=4{\cdot}2$, $D=4{\cdot}7$; while a further sample centrifuged at 20–22° gave higher values, $s=4{\cdot}5$, $D=6{\cdot}0$, approximately those originally observed. The conclusion was drawn, therefore, that in the irradiation experiments the results were not affected by the rotation, and were due solely to the U.V. light.

The sedimentation constant of human serum albumin appears to be definitely lower at *p*H 7·4 than in the region of the isoelectric point, as can be seen by comparison of Series B with the unirradiated solutions of Series A (Table I). In the solution used for Series C, the change to *p*H 7·4 from the isoelectric point was made at a temperature which certainly did not exceed 15°; the solution was subsequently kept frozen and thawed only for removal of samples. In all the solutions of this series, whether irradiated or not, the temperature of which had never risen above 22°, the sedimentation constant was that of isoelectric serum albumin, $s=4{\cdot}5$–$4{\cdot}6$, also observed in Series A, instead of the lower value which would be expected from the increase in *p*H; for the unirradiated solutions of this group, the spreading coefficients, too, were the same as in Series A, $D=6{\cdot}0$–$6{\cdot}6$. The lower values appeared only in the two samples which had been heated to 56° and 25–26° respectively. It is believed, therefore, that whatever the nature of the change producing these differences, it must occur at a negligible rate below 22–23°, but rapidly at 25° and above. In view of the very high temperature coefficients of many protein reactions, such a hypothesis is not theoretically unreasonable.

At *p*H 9·5 the molecular weight of lactoglobulin is the same as at the isoelectric point, but both the sedimentation constant and diffusion constant are lower by about 6% [Lamm & Polson, 1936]; this difference has been attributed to a change in the shape of the molecule, resulting in a higher frictional constant. Human serum albumin probably also acquires a higher frictional constant without change in molecular weight when brought to *p*H 7·4 from the isoelectric point, either because of a change in shape or because of a change in the degree of hydration.

While the values for the sedimentation constant and spreading coefficient for Series A are in good agreement with those of the unheated solutions in Series C, those of Series B, instead of agreeing with those of the heated solutions in Series C, lie intermediate between them and the isoelectric values. A possible reason for this discrepancy may be the greater age of the stock solution used for Series B, but the more probable explanation is the greater concentration of the heated solutions in Series C. The diffusion constant of serum albumin near the isoelectric point is substantially independent of concentration up to 2% [Lamm & Polson, 1936]; but it is possible that at *p*H 7·4, where the frictional constant is higher, increased concentration may more definitely repress both sedimentation and diffusion.

Summary

1. The effect of U.V. irradiation on 0·8% solutions of purified human serum albumin at *p*H 5·4 (1% NaCl) at about 30°, and at *p*H 7·4 (*M*/15 phosphate buffer) at 30 and 0°, was studied by means of sedimentation velocity and light absorption measurements.

2. Irradiation caused increased light absorption at both *p*H, general for wave-lengths longer than 2400 Å., but greatest at the protein band; the increase was greater at *p*H 5·4 than at *p*H 7·4. At *p*H 7·4, irradiation at 0° caused more increase for wave-lengths above 3000 Å., but less increase for wave-lengths below 3000 Å., than at 30°; after prolonged standing in the cold, the two gave identical spectra.

3. The results indicate that irradiation causes the liberation of substances of small molecular weight, and that it also leads to ill-defined photo-oxidation reactions which result in increased light absorption. It is possible that these reactions may involve both the albumin residues and the low-molecular cleavage products.

4. Though visible coagulation occurred only at *p*H 5·4, irradiation caused aggregation of the protein at both *p*H, and gradual reduction in the concentration of unchanged albumin. Aggregation was less pronounced at *p*H 7·4 than at *p*H 5·4, and a larger concentration of light-denatured molecules remained unchanged with respect to sedimentation constant. In the absence of a visible precipitate, irradiation led to the formation of successively heavier soluble aggregates, while when visible coagulation occurred the solution was impoverished with respect to soluble aggregates of large size.

5. A correlation was observed between increase of light absorption and decrease in concentration of unaggregated albumin in the irradiated solutions.

6. The sedimentation constant of the unaggregated protein in the irradiated solutions remained essentially that of native albumin.

7. Three hours' irradiation with soft X-rays (29,000 r.u.) caused no detectable change in the sedimentation behaviour or the absorption spectrum.

8. The sedimentation constant of serum albumin is lower at *p*H 7·4 than near the isoelectric point; the change (probably in the shape or degree of hydration of the molecule) which causes this difference, appears to be prevented at low temperatures and to occur rapidly only at temperatures above 23°.

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II. A STUDY OF THE PASSAGE OF FATTY ACIDS OF FOOD INTO LIPINS AND GLYCERIDES OF THE BODY USING DEUTERIUM AS AN INDICATOR

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It was shown by Schoenheimer & Rittenberg [1935] that fat containing deuterium when fed to mice may subsequently be found in both the "organ fat" and the "body fat" of the animals. The present authors [1936], after administering an oil containing D to a rat for 7 days, also showed that the lipins of the liver and kidney contained fatty acids in which D was present. This suggests that the lipins may play an active part in fat metabolism and not merely a passive role as constituents of protoplasm which are important because of their physical properties.

Sinclair [1935] has come to a similar conclusion as a result of experiments in which the administration of a fat containing elaidic acid led to the appearance of this acid in the lipins of liver and muscle. In Sinclair's experiments one day was the shortest period that was allowed to elapse after the fat feeding before the organs of the animals were taken for examination. It appeared to us that a shorter period than this might yield results of interest and, further, the greater ease with which D can be detected as compared with elaidic acid made its use especially advantageous for this type of work.

Experimental

The "deutero-fat" was obtained by partially "deuterating" pure unboiled linseed oil with 100% deuterium (electrolytic) using palladium black as catalyst. About 100 g. oil were treated at a time, the gas (in glass bulbs) was initially at atmospheric pressure, and the apparatus was entirely of glass with the exception of one short connexion of flexible metal tubing joined to the glass by piceined cones. The oil was heated electrically to about 70° and shaken mechanically during the reaction, the progress of which was observed manometrically. It was found possible to introduce D to the extent of 4–5 atoms % of the H present without solidifying the fat or making it unpalatable to the rats.

Groups of 3 or 4 rats were used according to the size of the animals. The total weight of the group was usually about 1 kg. They had been kept on the ordinary laboratory diet before the experiment. Each group was starved for a day before receiving the food containing "deutero-fat". This consisted of 4 g. crushed dog biscuit, moistened with 10 ml. water, and in this 1·9 g. of the deutero-fat were incorporated. The food was usually well taken and was eaten within 3 hr. At the appropriate time (6, 10 or 24 hr.) after giving the food the animals were stunned and killed by bleeding. The blood was collected with addition of oxalate and centrifuged to separate corpuscles and plasma. The liver, kidneys and brain were

removed and a sample of adipose tissue from the abdomen was taken. The tissues or organs of the whole group were worked up together. The adipose tissue was heated with about its own wt. of 50 % KOH and 10 ml. alcohol until saponification was complete. The alcohol was then removed on the water bath, the soaps taken up in water and acidified with dilute H_2SO_4. The liberated fatty acids were taken up in light petroleum and a portion of the solution evaporated. The last traces of the solvent were removed by heating to 100° under reduced pressure in an atmosphere of H_2 for $\frac{1}{2}$ hr. The liver, kidneys, brain, blood corpuscles and blood plasma were dealt with as follows. The organs after chopping with a razor were ground up in a mortar and extracted in the cold for 24 hr. with 4–5 times their wt. of alcohol—the blood corpuscles and blood plasma were added directly to the alcohol. The extraction was repeated with absolute alcohol for at least another 24 hr. The tissue substance after removal of the 2nd extract by filtration was dried in a vacuum desiccator over H_2SO_4 and then extracted with ether for 8 hr. in a Soxhlet apparatus. The two alcohol extracts were evaporated to dryness under reduced pressure in a current of H_2 and the residue extracted with dry ether. This extract was added to the Soxhlet extract and the whole distilled to remove the ether. With the liver it was found convenient at this stage to continue with only one-fourth of the extract. The residue containing all the ether-soluble substances of the organ (or blood fraction) was dissolved in 4 ml. dry ether and precipitated with 20 ml. acetone. The precipitate was filtered off, washed with acetone-ether and dried in a vacuum desiccator. It was then taken up in dry ether and the solution evaporated in a weighed flask. The acetone-soluble material was recovered in a similar manner by distillation from a tared flask. In these two fractions the D content was estimated as described below. The acetone-insoluble fraction consists essentially of the lipins of the tissue, and the acetone-soluble fraction of the glycerides and sterols.

Combustion technique. The commercial O_2 used in the combustions was known to be liable to contain traces of oil, and for this reason, as well as on general grounds, the dried gas was purified by submitting it to exactly the same treatment as it would subsequently encounter in the combustion tube, i.e. it was passed through a duplicate tube similarly packed and heated and was then dried before entering the combustion tube proper. In the earlier combustions Pregl's "universal filling" was used in supremax glass tubes. The too hygroscopic lead peroxide was omitted since interchange of water from one combustion to another was not permissible, but otherwise, as regards temp., rate of O_2-flow etc., the essential conditions of the Pregl technique were observed, though the heating was electrical. Later it was found convenient to use quartz combustion tubes, omitting the lead chromate from the filling and raising the temperature somewhat (750–800°). The tubes (Fig. 1) were wound directly with nichrome ribbon and then boxed in with thick alundum cement. The current (A.C.) was controlled by rheostat and ammeter and the temperature inside the lagging observed by means of a thermocouple.

Since, of the products of combustion, only the water was required and this in the free form, the emergent gases were passed through a glass spiral (*H*) immersed in a "slush" of solid carbon dioxide and alcohol. (A similar cooled spiral followed by two U-tubes packed with $Mg(ClO_4)_2$ was used for drying the O_2 stream between the two combustion tubes.) There were no rubber connexions, a pieceined ground stopper (*G*) permitted the introduction of the Pt boat containing the sample for analysis, and on completion of the combustion, glass taps served to cut off the O_2 stream (at *A*) and its exit (at *B*) and to connect the combustion tube (at *C*) to the Hg vapour pump. Moderate evacuation then sufficed to enable the water

sample to be rapidly distilled through a large tap (*D*) into a separate compartment of the apparatus where it was condensed in a detachable "water vessel" of the type shown (*E*), and then resublimed from $-15°$ to $-30°$ into a second vessel (*F*) under a better vacuum.

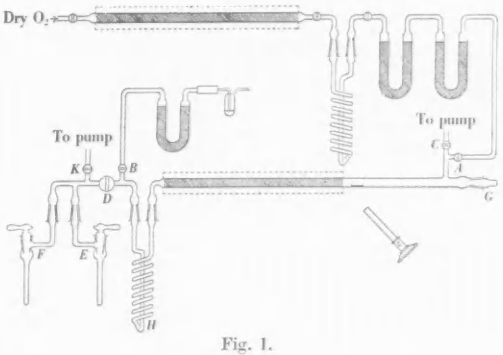

Fig. 1.

Purification of water from combustion. About half of the samples dealt with were known to contain N and the rest might contain small amounts, but since this was in the form of substituted NH_3 it was not at first thought necessary to supplement the filling by Cu for the decomposition of oxides of N. However, a peculiarity in the behaviour of the collected water during sublimation led to tests which revealed the presence of HNO_3 in appreciable quantities in the collected water. It was therefore decided to use the Cu catalyst, but to do so in a separate apparatus, so as to avoid the necessity of frequent reduction of the Cu. This apparatus was essentially similar to the main combustion apparatus (lower part of Fig. 1) without the provision of the O_2 stream, with a ground joint for the attachment of a "water vessel" in place of *G*, a narrow U-tube in place of *H*, and the Cu catalyst alone as filling. The latter was maintained (electrically) at a mild red heat and the whole apparatus thoroughly evacuated before each experiment. The water sample from a combustion attached at *G* was first cooled to $-80°$ while the evacuation was completed, and then (taps *C* and *D* being closed) allowed to warm up in stages to room temp. while the U-tube was cooled. The controlled distillation over the heated catalyst was accelerated in its later stages by pumping off permanent gases through *D* and *K*. The purification of the water sample was then completed by a double vacuum sublimation (between $-15°$ and $-30°$) to the receivers *E* and *F*. As a check the water samples were always tested for neutrality after the densities had been determined.

Density determination. The purified water samples were generally about 10 mg. but in several cases as small as 4–5 mg. Their densities were determined by the method of Gilfillan & Polanyi [1933] to a precision of one part in 100,000, and the atomic % of D calculated to 0·01 %. As a check on the combustion technique samples of ox-liver lecithin free from D were similarly treated, one such control being inserted after every two of the ordinary combustions. Similar controls were used initially in perfecting the technique and afterwards—throughout the recorded experiments—they yielded water samples of normal density within the precision of measurement.

Results

The % of the "glyceride" and "lipin" fractions obtained from the organs or tissues and the D contents of these fractions are given in Tables I and II respectively. The % figures for both fractions are reckoned on the wt. of the fresh tissue except with the blood corpuscles and plasma, which are based on 100 ml. The D figures represent atoms % D in the crude "glyceride" and "lipin" fractions. The fat administered contained 4·87 atoms % D.

Table I. *Glyceride fractions*

	Glyceride %			Atoms % D in glycerides		
Organ or tissue	6 hr.	10 hr.	24 hr.	6 hr.	10 hr.	24 hr
Liver	1·26	1·41	1·55	0·86	0·67	0·30
Kidney	1·27	1·43	—	0·12	0·22	0·20
Brain	2·98	2·87	2·50	0·01	0·05	0·025
Blood corpuscles	0·32	0·43	0·50	0·67	0·12	0·17
Blood plasma	0·23	0·28	0·19	1·26	0·64	—
Adipose tissue	—	—	—	0·06	0·03	0·13

Table II. *Lipin fractions*

	Lipin %			Atoms % D in lipins		
Organ or tissue	6 hr.	10 hr.	24 hr.	6 hr.	10 hr.	24 hr.
Liver	3·97	4·42	4·11	0·47	0·52	0·42
Kidney	—	2·85	2·79	0·16	0·14	0·17
Brain	4·0	—	4·39	0·004	0·08	0·03
Blood corpuscles	0·59	0·40	0·26	0·12	0·10	—
Blood plasma	0·07	—	0·08	0·12	0·47	—

Discussion

Acetone precipitation does not produce a sharp separation of lipins from glycerides so that the experiments give comparative rather than strictly quantitative indications of the distribution of the deutero-fatty acids between glycerides and lipins after absorption. Perhaps the figures of greatest interest are those obtained 6 hr. after administration of the fat. At this time the fatty substances of all the tissues examined showed the presence of D. As would be expected the highest % D was found in the glycerides of the blood plasma and following these in descending order come those of liver, blood corpuscles and kidney. In the brain and adipose tissue D was only just detectable. The % D in the liver lipins and glycerides was very much greater than in those of the kidney, which accords with the generally accepted views on the special part played by the liver in fat metabolism. A comparison of the % D in the various organ fats with that in the fat administered shows that at the end of 6 hr. about 26 % of the plasma glyceride, 18 % of the liver glyceride and 2·5 % of the kidney glyceride was derived from the deutero-fat administered. This indicates a selective intake of fat from the blood by the liver soon after its absorption. The lipins of the liver also participate in this phenomenon. Although the % D in the liver lipins is lower than that in of the glycerides, if one takes into account the smaller proportion of fatty acids which they contain (65 %) it is seen that about 14 % of the lipin fatty acids had come from the deutero-fat of the food.

The figures obtained after 10 hr. show a decline in the D content of the blood fat. This might be expected from previous observations on fat absorption. Owing to technical difficulties the 24 hr. figures for the plasma and corpuscles were not

obtained. In the liver there is a gradual decline in the % D in the glycerides during 24 hr. but much less change in that of the lipins. In the kidney the glyceride figures at 10 and 24 hr. are about the same and higher than the 6 hr. value, whereas the lipin D does not change significantly. The kidney, therefore, does not appear to respond to the changes in blood fat either as rapidly or to the same extent as the liver. On the whole these results suggest a specially active participation in fat metabolism by the lipins of the liver. Artom *et al.* [1937] have reached a similar conclusion from experiments in which radioactive P was used to trace the entry of H_3PO_4 into lipins. The lower % D in the plasma lipins in comparison with those of the liver at the end of 6 hr., renders it unlikely that the liver lipins were not synthesized in the liver but merely taken up from the blood.

The D content of the adipose tissue was never very marked and this was to be expected because of the diluent effect of the comparatively large amount of adipose tissue fat in the animal compared with that (1·9 g.) which was administered.

The brain in all the experiments contained only traces of D in its lipin and glyceride fractions. This does not suggest any rapid exchange in this organ between its lipins and the blood fat. It confirms observations made by Hahn & Hevesy [1937], who found a definite but small uptake of labelled P by brain lipins an hour after the injection into rats of Na phosphate containing the radioactive isotope of phosphorus.

SUMMARY

1. Rats were fed with a fat containing 4–5 atoms % deuterium and the distribution of the "deutero-fatty acids" in "lipin" and "glyceride" fractions of liver, kidney, brain and blood determined 6, 10 and 24 hr. after.

2. After 6 hr. D was present in considerable amount in plasma glycerides, liver glycerides and liver lipins. There was much less in the lipoid fractions of the kidney and plasma and the D was only present in traces in brain and adipose tissue.

3. The D in the liver glycerides decreased more rapidly in 24 hr. than that of the liver lipins. In the lipoid fractions of kidney and brain there were no notable changes in % D between 6 and 24 hr.

4. The results suggest that liver lipins may play a very active part in fat metabolism.

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III. THE CALCIUM REQUIREMENT OF OLDER MALE SUBJECTS

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It is now generally accepted that Ca is an important essential in nutrition. It is believed, moreover, that, in most countries of the world, large sections of the population would be more adequately nourished were they to ingest more Ca. This applies to Great Britain [Orr, 1937], India [McCarrison, 1932–3], U.S.A. [Sherman, 1920, 2], China [Kung & Yeh, 1937; Liu *et al.* 1937], and figures given by Rubner [1920] leave little doubt that it applies also both to Germany and to Japan.

Orr & Clark [1930] showed that the health of school children could be improved and that their growth could be accelerated by giving them a supplement of whole or separated milk. More recent experiments by Gaunt *et al.* [1938] indicated that the benefits resulting from the addition of milk and greenstuffs to poor-class diets were due mainly to the Ca and P which these additional foodstuffs contain. Aykroyd & Krishnan [1938] have found the health and growth rates of Indian school children to be improved by a daily ration of Ca lactate.

The food shortage in Germany and Austria during the world war showed very clearly the result of a widespread lack of Ca and P. Hundreds of cases of "hunger osteomalacia" occurred. Alwens [1919], after careful clinical investigation of 29 such cases, mostly old subjects, in Frankfurt, concluded that they were suffering from osteoporosis due to protein, Ca and P deficiency. Dalyell & Chick [1921] investigated over 100 cases and also thought "hunger osteomalacia" to be a nutritional disease of the skeleton. Hume & Nirenstein [1921] showed that the incidence of the disease was highest in winter when the diet was poorest and that there was a remarkable increase of incidence with age. Of 131 subjects examined 115 were over 40 years of age and one-third were between 60 and 70.

Such observations show the importance of the hypothesis of Meulengracht & Meyer [1936] who, after intensive investigation of 5 cases of senile osteoporosis, came to the conclusion that long-standing Ca deficiency was the main factor in the genesis of this disease. Meulengracht [1938] has again advanced this hypothesis in connexion with a case of senile osteoporosis produced by repeated purgation with Na_2SO_4. Leitch [1936–7] supports Meulengracht's opinion as to the relation of Ca deficiency to senile osteoporosis.

Before the effects of a long-standing deficiency of Ca can be properly evaluated it is necessary to ascertain the Ca requirement of older subjects. A decrease of requirement with age would offset, to some extent, the effects of low intake. It is also of importance to find out whether, after depletion of these elements, the older organism will store additional Ca and P in the same way as the young adult. The following balance experiments were therefore undertaken to decide these points.

Plan of experiments

The experiments were all done in the metabolic ward of the Aberdeen Royal Infirmary. Balances were determined on 10 subjects, mostly old or middle-aged. A preliminary period on a low Ca diet was followed by one on a higher intake. A specially trained sister supervised the preparation and consumption of the diets and the collection of all excreta.

In all cases balances were determined every 3 days but the data for the first 3 days on the low Ca diet were not included in the results. Both low and high Ca periods lasted at least 9 days.

Duplicate diets were analysed for total Ca and P in 3-day lots; urine and faeces were similarly analysed every 3 days. The methods of analysis were, with minor modifications, those recommended in *Technical Communication* No. 9 of the Imperial Bureau of Animal Nutrition [Godden, 1937], i.e. P was estimated by the method of Fiske & Subbarow [1925], and Ca by the method of McCrudden [1909; 1911]. Ca in urine was determined by direct precipitation. The urine was, if necessary, deproteinized prior to precipitation, acetic acid being used for the purpose. Ca in ashed diet and in ashed faeces was determined by precipitation of the oxalate in the presence of sodium acetate. Dry ashings preceded all P determinations; in the case of diet and faeces, $Mg(NO_3)_2$ was added and with urine Ca acetate was employed. All ashings were completed in an electric muffle.

Male outpatients from the diabetes and obesity clinic of Dr A. Lyall were used; they varied in age from 32 to 70 and were kept in bed during the whole period of the experiment. Blood Ca and P, and serum phosphatase of all patients were within normal limits.

The dietary history of these patients for the previous 6 months was known, including their Ca and P intakes. The composition of the diet during the preliminary low Ca period was adjusted to a similar level. The diets used in the balance experiments consisted entirely of normal foodstuffs and the Ca and P contents in the second period were raised solely by addition of foods rich in these materials. In cooking the food, distilled water was used and all such water was included in the diet, for example in soups. Eggs were fed always without shells since some patients were found to eat egg-shells. Only on very few occasions did patients reject food; in all such cases a similar rejection was made from the duplicate diets. The composition of the two diets is given in Table I.

Table I. *Composition of diets I and II expressed as daily intakes*

	Diet I	Diet II
Calories	1493	1509
Protein	59	85
Carbohydrate	141	146
Fat	77	65
Ca	0·53	0·88
P	0·85	1·23
Ca/P ratio	0·62	0·72
Ca as % of dry wt. of diet	0·184	0·273
P as % of dry wt. of diet	0·294	0·381

Results

All the data relevant to the Ca and P retentions of the subjects investigated will be found in Table II. On the lower Ca level it will be seen that four of the subjects were in positive Ca balance and five in negative, while the remaining one

Table II. *Summary of balance experiment results*

Subject no.	Age of subject	No. of 3-day balances	Ca (mg. per day) Intake	Output Urine	Output Faeces	Output Total	Balance
1	53	7	510	130	383	513	− 3
		1	819	157	532	689	+130
2	45	5	491	141	370	511	− 20
		4	861	206	539	745	+116
3	66	3	503	192	382	574	− 71
		3	869	195	605	800	+ 69
4	53	3	503	199	251	450	+ 53
		3	869	266·5	463·5	730	+140
5	42	3	510	156	531	687	−177
		3	942	201	472	673	+269
6	32	3	510	165	370	535	− 25
		3	942	214	544	758	+184
7	69	3	567	77	450	527	+ 40
		3	896	100	505	605	+291
8	65	3	567	65	281	346	+221
		3	896	104	595	699	+197
9	65	3	544	49	407	456	+ 88
		3	849	77	660	737	+112
10	40	3	544	542	136	678	−134
		3	849	544	293	837	+ 12
Average of the 10 subjects			524	172	356	528	− 4
			879	206	521	727	+152

Subject no.	Age of subject	No. of 3-day balances	P (mg. per day) Intake	Output Urine	Output Faeces	Output Total	Balance
1	53	7	879	832	200	1032	−153
		1	1210	960	256	1216	− 6
2	45	5	851	814	214	1028	−177
		4	1286	987·5	283·5	1271	+ 15
3	66	3	839	746	245	991	−153
		3	1201	807	365	1172	+ 29
4	53	3	839	836	135	971	−132
		3	1201	1083	238	1321	−121
5	42	3	840	833	242	1075	−235
		3	1226	964	237	1201	+ 25
6	32	3	840	760	208	968	−128
		3	1226	962	277	1239	− 13
7	69	3	845	737·5	229·5	967	−122
		3	1311	883	249	1132	+179
8	65	3	845	630	193	823	+ 22
		3	1311	664	428	1092	+219
9	65	3	858	699	227	926	− 68
		3	1149	691	428	1119	+ 30
10	40	3	858	1015	125	1140	−282
		3	1149	977	210	1187	− 38
Average of the 10 subjects			849	790	202	992	−147
			1227	898	297	1195	+ 32

was in equilibrium. The average intake of the whole group while on the lower Ca diet was 524 mg. Ca per day, and the average output was 528 mg. per day, i.e. the subjects as a group were practically in equilibrium.

The P balances on the lower Ca diet were negative in every case except no. 8. The average P intake was 849 mg. and the average output 992 mg. per day.

At the higher Ca level all the subjects without exception went into positive balance. The average Ca intake of the group was 879 mg. per day and the average output 727 mg. per day. In all but one case 900 mg. Ca per day would certainly more than suffice to keep these subjects in Ca equilibrium. The time necessary to attain equilibrium at the higher level of intake is evidently considerably longer than the duration of the present experiments.

On the higher Ca intake the P intake was also higher and the average P balance of the group was positive. The average P intake was 1227 mg. per day and the average output 1195 mg. per day. Individually the subjects were all very nearly in equilibrium except no. 4 who was in a distinct negative balance and nos. 7 and 8 who were in strongly positive balance. At the higher level of P intake no. 4 was still losing P despite his having been in no way unusual at the lower level. At both levels of intake he was retaining Ca.

All the subjects excreted P chiefly in the urine at both intake levels, while Ca was excreted chiefly in the faeces. No. 10 was unusual in excreting much more Ca in the urine than in the faeces. On the lower Ca diet his output in the urine alone almost exactly equalled the total Ca ingested. This abnormality suggested further investigation which is now being carried out.

Discussion

The figure here obtained for the Ca requirement of older men agrees well with the estimate of 450 mg. per person per day given by Sherman [1920, 1]. It also agrees well with that of Leitch [1936–37] for adult, non-pregnant, non-lactating women; she found a requirement of 550 mg. per day. Brull [1936], from subjects who were in negative balance, arrived at a similar estimate, though it is doubtful if the output of a subject in negative balance is a true index of his requirement. The data of Kelly & Henderson [1929–30] seem to indicate retention at a level considerably lower than the one here found. As their subjects were negro prisoners in Africa these better retentions may have indicated that the skeletons of their subjects were partly depleted of Ca and P before coming under investigation, since Rottensten [1938] has shown that retention may be more efficient in such circumstances. The subjects had been subsisting for some time on the prison diet containing 300 mg. Ca and 2390 mg. P per day.

The P requirement of the older adults here considered is approximately 1200 mg. per day. This figure exceeds the estimate of 900 mg. given by Sherman [1920, 1]. Requirements of this order have, however, frequently been reported, as shown in the review of P metabolism by Forbes & Keith [1914]. Figures quoted by Orr & Clark [1930] are also of this order.

Both diets were of normal composition and it is of interest that the Ca/P ratio was so low in each case. This is, however, apparently usual in human dietaries since many diets determined by survey at the Rowett Research Institute have similarly low ratios. Little can be argued from the ratio in the present experiments since it is virtually the same in both diets.

It may appear strange that subjects who were apparently in equilibrium on the lower intake should have gone into marked positive balance when the intake was increased. This is almost certainly due to the fact that the lower level

represents the lowest for maintenance and allows no margin against unforeseen Ca loss. It had been noted that at this level an attack of diarrhoea might cause a considerable loss of Ca. A study of Table II shows that at the lower level there were as many negative as positive balances, while at the higher level they were all positive. This increase in or change to a positive balance probably represents the making good of small losses of Ca incurred over a considerable prior period.

The Ca requirements of older males are much the same as those of younger adults: the effect, therefore, of long subjection to a diet low in Ca is not offset by increasing age. The older organism likewise resembles the young adult in that it readily retains Ca and P after depletion. The possible bearing of these facts on the genesis of senile osteoporosis has been discussed in the introduction.

Summary

Ca and P balances determined on 10 older males are reported, and the possible bearing of the results on the aetiology of senile osteoporosis is discussed. The results indicate that Ca and P equilibrium may be attained with 520 mg. Ca and 1200 mg. P per day.

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IV. ENZYMIC METHODS FOR THE PREPARATION OF ARGININE AND ORNITHINE

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THIS paper deals with two examples of the application of enzymes to preparative purposes: (1) the use of "trypsin" in the preparation of arginine, and (2) the use of arginase with urease in the preparation of ornithine. The first, while not devoid of practical utility, is described chiefly as a demonstration of the existence of substantial amounts of free arginine in pancreatic digests. The second is offered as a wholly satisfactory method for the preparation of ornithine, possessing certain advantages over any hitherto described.

1. *Isolation of arginine from pancreatic digests*

Some years ago Dauphinee & Hunter [1930] showed that digestion with commercial trypsin would split off, in a form accessible to the action of arginase, about 80% of the total potential arginine of certain proteins. The question, whether the arginase-susceptible material thus liberated is arginine itself or an arginine complex, was at that time left open. Later (unpublished) observations by Mr J. W. Chambers and the writer have indicated that it is probably a mixture, in which demonstrably free arginine forms at the beginning a relatively small, but, as proteolysis proceeds, an increasingly large, proportion of the whole. When, after about two weeks of digestion, that whole has reached its maximum, as much as 80 or 90% of it may be simple arginine.

This fact may be utilized in the preparation of arginine from protein by substituting for the usual acid hydrolysis the milder action of the pancreatic enzymes. The substitution has drawbacks, but also some advantages. Its drawbacks are (1) that, since the enzyme hydrolysis is incomplete, the yield of arginine is correspondingly reduced, and (2) that the need to separate the free arginine from arginine-containing complexes calls for a double precipitation with flavianic acid. The advantages are a simplification of the initial manipulations, and, more important, the avoidance of the partial racemization, which is produced by the long-continued action of boiling acids. The enzymic method leads, it will be shown, to a product of exceptional optical purity. How far this may compensate for the lower yield will depend upon circumstances.

The procedure is applicable to any protein which contains a relatively high proportion of arginine, and which at the same time is hydrolysable to a sufficient extent by the pancreatic enzymes. The detailed description which follows is adapted specifically to the case of gelatin.

250 g. air-dry gelatin are dissolved in 2500 ml. water at 37°. By appropriate addition of NaOH the solution is made definitely alkaline to phenolphthalein, and it is then treated with 10 g. of an active commercial trypsin suspended in about 50 ml. water. The mixture is covered with a layer of toluene and incubated at 37° for 15 to 20 days, i.e. until the "free" arginine, as determined by arginase, has reached a maximum.

The digest is now acidified with 5 *N* H_2SO_4 (at the rate of 2 ml. per 100), boiled, filtered from coagulated protein, heated almost to the boiling point, and treated with 450 ml. of a 20 % solution of flavianic acid. After a day or two in the ice-chest the granular yellow precipitate of crude arginine flavianate is collected on a large fritted glass filter (porosity 3), and washed with a cold 0·1 % solution of flavianic acid in 0·025 *N* H_2SO_4.

For purification the flavianate is suspended in about 1800 ml. water, and brought into solution with the minimum amount (about 20 ml.) of 5 *N* NaOH. The solution is diluted to about 2500 ml., heated again just short of boiling, and treated with a mixture of 50 ml. 5 *N* H_2SO_4 and 225 ml. (half the amount used in the first precipitation) of 20 % flavianic acid. In a minute or two the arginine flavianate begins to separate from the hot solution, this time in the form of large glistening iridescent orange-yellow leaflets. The mixture is allowed to cool slowly to room temperature, and is then set in the ice-chest for at least 24 hr. The precipitate is finally collected and washed in the same way as the first.

Precipitation with flavianic acid in the heat, as recommended, has several advantages. Not only does it yield the product in a form especially convenient for further manipulation, but it affords also a certain criterion of its purity; for if, under the conditions described, the flavianate assumes a granular form, it is almost certainly still contaminated.

From the purified arginine flavianate the arginine may be recovered by any of the standard methods, but the procedure adopted in this laboratory is the following. The flavianate is suspended in 500 ml. water and brought into solution with the minimum amount (about 12 ml.) of conc. NH_3. The solution is diluted to about 800 ml. and treated with a definite excess of cold saturated baryta water. The precipitate of barium flavianate is filtered off under suction, and washed thoroughly with very dilute baryta. From the mixed filtrate and washings the barium is precipitated quantitatively with H_2SO_4, and the residual flavianic acid is removed by treatment with the minimum amount of norite. $BaSO_4$ and norite are filtered off together. The colourless filtrate is freed of NH_3 by evaporation to a small volume, is then neutralized exactly with HCl and is finally evaporated to dryness, yielding directly a crystalline mass of arginine hydrochloride. This product, weighing as a rule between 15 and 17 g., is purified by dissolving it in a little water, rubbing up the solution with small amounts of alcohol until crystallization sets in, adding then about two more volumes of alcohol, and cooling overnight in the ice-chest.

In several experiments with this procedure the final yield of pure arginine hydrochloride has varied between 13·5 and 15·6 g. A representative sample gave by Kjeldahl 26·48 % N ($C_6H_{14}O_2N_4.HCl$ requires 26·60 %), and left on incineration less than 0·1 % of ash. In the presence of seven extra molecules of HCl it had $[\alpha]_D^{22°} = 22·19°$ ($c = 11·47$; $l = 2·2$ dm.; $\alpha = +5·60°$). The highest value hitherto recorded for the specific rotatory power of arginine hydrochloride under the conditions specified is +21·95° [Hunter & Morrell, 1922; Hunter, 1929]. The product of the enzymic method of preparation was therefore *l*(+)-arginine of at least as great a degree of optical purity as has so far been attained. The experiments gave no indication whatever of the production of inactive arginine, such as has been described by Kutscher [1899; 1901] as occurring during the tryptic digestion of fibrin.

The gelatin used in the present work contained, according to the arginase method, 7·73 g. of potential arginine per 100 g. (air-dry). The yields of recrystallized hydrochloride mentioned above represent therefore only from 58 to 67 % of the possible maximum and roughly three-fourths of the amount recoverable

from a total hydrolysate by the excellent method of Cox [1928]. Of course, it has to be remembered that in the enzyme hydrolysates only 80 % or so of the total arginine had been so far liberated as to be accessible to the action of arginase [Dauphinee & Hunter, 1930]. Of this fraction the proportion finally accounted for as pure hydrochloride is from 73 to 84 %. This result was attained by procedures which, on the one hand, would be unlikely to bring about more than a minimal hydrolysis of arginine peptides, and which, on the other, certainly involved some losses. It seems therefore not unreasonable to conclude that in the final stages of pancreatic digestion at least 80 or possibly as much as 90 % of the arginase-susceptible material is actually free arginine.

The literature contains a few previous reports of the isolation of arginine in substance from pancreatic digests [Kossel & Mathews, 1898; Kutscher, 1898; 1899; 1901]; but the yields (never exactly stated) in these earlier experiments appear to have been relatively small.

2. *Preparation of ornithine*

The methods heretofore proposed for the preparation of ornithine from arginine have varied with respect to (1) the agent employed to hydrolyse the arginine, (2) the procedure followed in separating the ornithine from urea and from the hydrolysing reagent and (3) the form in which the ornithine has been finally isolated. For the hydrolysis use has been made of $Ba(OH)_2$ [Schulze & Winterstein, 1898; Bergmann & Zervas, 1926; Kurtz, 1938], NaOH [Boon & Robson, 1935] and arginase [Kiesel, 1911; 1922; Felix & Röthler, 1925; Vickery & Cook, 1931]. To separate the ornithine Schulze & Winterstein [1898] employed benzoylation, Bergmann & Zervas [1926] and Boon & Robson [1935] condensation with salicylaldehyde, and the other authors (Kurtz excepted) precipitation with phosphotungstic acid. As a rule the base has ultimately been converted into the hydrochloride; but Kiesel [1911] isolated it as carbonate, Felix & Röthler [1925] and Vickery & Cook [1931] as picrate, Kurtz [1938] as sulphate.

The earlier methods, whatever combination of procedures they represented, gave very unsatisfactory yields. Schulze & Winterstein [1898] appear to have obtained from arginine at most 30 % of the theoretical amount of ornithine; and no better result was claimed for any of the alternative procedures published prior to 1931. Parenthetically it may be mentioned that in 1929 Mr H. B. Collier, working under the writer's direction, obtained ornithine hydrochloride in yields up to 45 % by the action of arginase upon arginine, followed by condensation of the product with salicylaldehyde in the presence of $Ba(OH)_2$. Collier's results were left unpublished because of uncontrollable irregularities encountered in the application of the salicylaldehyde method. Vickery & Cook [1931] also had difficulties with this method, but by the use, under special conditions, of phosphotungstic acid obtained from an arginase digest ornithine (as dipicrate) corresponding to 55 % of the arginine taken.

Really satisfying yields ("approximately theoretical") were first attained by Boon & Robson [1935], who recommended hydrolysis (of carbamido-arginine) with 20 % NaOH, separation of ornithine as the barium salicylidene derivative, and conversion of the latter into the hydrochloride. In the yet more recent method of Kurtz [1938] carbamido-arginine is treated with baryta under conditions which not only split it into ornithine and urea, but which also ensure the destruction of the latter. Since the baryta itself is readily removed, preliminary separation of the ornithine can be dispensed with, and it is precipitated directly (as sulphate) by alcohol. Yields range from 76 to 82 %.

The method of Kurtz is the simplest yet proposed; but, like all methods depending on prolonged hydrolysis by alkalis, it has the drawback of yielding a product which is either partially or completely racemized. If the natural dextrorotatory ornithine is required, it is desirable, if not imperative, to resort in the first place to enzymic hydrolysis, and to restrict to the necessary minimum any subsequent exposure of the product to the action of alkali. Now for the successful application of the enzymic method two points are of practical importance. In the first place, in order to effect a complete hydrolysis of the arginine (which previous enzyme methods have evidently failed to do) it is necessary to use a large amount of arginase—about 1000 units [Hunter & Dauphinee, 1930] per g. arginine. In the second, it is obviously desirable to introduce along with the enzyme as little extraneous and inert material as possible. From this point of view a crude glycerol extract of liver is a quite unsuitable reagent. Later there is described the preparation from aqueous liver extracts of a concentrated arginase solution containing in 1 ml. from 250 to 380 units of the enzyme but only a fraction of the solid material with which that enzyme was originally associated. Under appropriate conditions from 2·5 to 4 ml. of such a solution amply suffice for the complete hydrolysis of 1 g. arginine, yet introduce little that cannot afterwards be removed by heat coagulation. There is therefore a possibility of preparing digests, which contain hardly anything in solution but an ornithine salt and urea.

It was thought at first that the separation of these two substances from one another might be accomplished in a very simple way by taking advantage of their greatly differing solubilities in alcohol. Had this proved possible, the steps necessary for the isolation of ornithine would have been few and straightforward. Unfortunately attempts to precipitate ornithine from the concentrated protein-free digest with alcohol, or to recrystallize from alcohol the solid residue left on evaporation, gave disappointingly small yields of a product which was always contaminated with urea. It remained necessary, therefore, either (*a*) to precipitate the ornithine first as phosphotungstate or other insoluble compound or (*b*) to destroy the urea. Since one of the objects sought was the elimination of the usual intermediate precipitation, the alternative adopted was the second; and in order to keep the whole method an enzymic one the agent chosen for the destruction of urea was urease. This can be quite conveniently applied in combination with arginase, so that the decomposition of urea may proceed *pari passu* with the production of ornithine. The alkaline reaction rapidly produced by the development of ammonium carbonate favours the action of the arginase without unduly depressing that of the urease. When the process is complete, both the ammonium and the carbonate ions can be got rid of by distilling the mixture with an excess of baryta. The barium having been in its turn removed, one is left with a solution of ornithine (or an ornithine salt), which can be freed from residual (mostly inorganic) impurities in the ordinary way.

The methods of preparation described below are based on the principles thus outlined.

(A) *Enzyme reagents required*

Arginase. A crude arginase extract is prepared according to the method of Hunter & Dauphinee [1930] with the single change that water is substituted for 75 % glycerol. One volume of this extract is mixed with 1·2 volumes of acetone. The flocculent precipitate is separated by centrifuging, and extracted with half the original vol. of water. Any insoluble material is filtered off, and the filtrate is treated again with 1·2 vol. of acetone. The second precipitate is taken up in

one-fourth of the original volume, and to this final solution cobalt chloride is added in the proportion of one drop of a 1 % solution for each 10 ml. The activity of the solution is determined by the method of Hunter & Dauphinee [1930]. It should contain the least 200, preferably between 300 and 400, units of arginase per ml.

Urease. 10 g. jack-bean flour are shaken for 10 min. with 100 ml. of 30 % alcohol, and the mixture is then filtered.

(B) *Preparation of ornithine monohydrochloride from arginine hydrochloride*

To a solution of 10 g. arginine hydrochloride in 250 ml. water there are added (1) the prescribed arginase solution in amount sufficient to provide 8000 units of the enzyme, and (2) 10 ml. of the urease solution. The mixture, protected by toluene, is incubated at 37°. At first faintly acid, it rapidly develops an alkaline reaction and a strongly ammoniacal odour. After 4 or 5 days it is made just acid to Congo red with 5 *N* H_2SO_4 (about 18 ml.), and is boiled to coagulate proteins. (Acidification may, if it is thought worth while, be preceded by a preliminary distillation *in vacuo* with alcohol; this effects the removal of a considerable proportion of the NH_3, and reduces correspondingly the subsequent consumption of H_2SO_4 and baryta.) The mixture is cooled, and treated with a considerable excess (about 225 ml.) of saturated baryta water. The precipitate of coagulated protein and insoluble Ba salts is filtered off and washed with dilute baryta water. Filtrate and washings are transferred to a large Claisen flask, and freed from NH_3 by distillation *in vacuo* at a temperature not exceeding 50°. Frothing, when it occurs, is controlled by liberal additions of alcohol, repeated, as often as may be necessary, until the evolution of NH_3 comes to an end. The distillation residue should then be negative to Nessler's reagent. If it is not, more baryta is added, and the distillation continued.

When one is certain that the ammonia has been completely removed, the residue is freed exactly from barium by H_2SO_4, and filtered. It should now be neutral or only slightly alkaline, and give no more than a feeble Sakaguchi reaction for arginine. Having, if necessary, been made exactly neutral with HCl, it is concentrated on the water bath to about 75 ml., decolorized by heating with a generous addition of norite, filtered, and evaporated to dryness. If the product becomes seriously discoloured during evaporation, the treatment with norite is repeated. There is thus obtained a crude ornithine monohydrochloride, nearly white, but contaminated as a rule with a considerable quantity of inorganic salts.

For its purification the crude material is dissolved, by prolonged boiling under a reflux condenser, in the smallest possible volume of 75 % alcohol. If necessary, the hot solution is rapidly filtered through a fritted glass filter. Crystallization begins almost immediately. After a day in the cold chamber the crystals are collected, and washed with a little 95 % alcohol. This first crop should weigh about 6 g., and should not contain more than 0·3 % ash. A second crop of equal purity is obtained by evaporating the mother liquors to dryness and again recrystallizing the residue from 75 % alcohol. In this way the yield may be increased to 7 g. or more.

The results of two experiments with this method, including the N (Kjeldahl) and ash contents of the products, are given in Table I. The lower yield of the first experiment is accounted for by the fact that in this, an early one, only one crop of crystals was collected. With a little experience it is easy to reproduce the improved results of the second.

(C) *Preparation of ornithine hydrochloride or sulphate from protein*

The success of the enzymic method as applied to arginine itself suggested that it might be possible by similar means to isolate ornithine directly from any protein hydrolysate reasonably rich in arginine. In this way one would not need first to prepare (or procure) pure arginine. This idea, in its first shape, was frustrated by the difficulty of separating the ornithine from the many other substances in the arginase-treated hydrolysate. In a modified form it proved to be entirely feasible. Neither arginase nor urease is inactivated by flavianic acid. All that is necessary therefore to obtain ornithine from a protein is to hydrolyse the latter, precipitate the liberated arginine once with flavianic acid, and treat the arginine flavianate in the same way as one would the hydrochloride. When this is done, the baryta used to liberate NH_3 serves also to remove the bulk of the flavianic acid. The ornithine may be isolated either as hydrochloride or as sulphate. In detail the procedure is as follows:

A convenient weight of some appropriate protein (say 200 g. gelatin or 50 g. protamine) is hydrolysed by boiling for 12 hr. with a ten-fold quantity of 20 % HCl. The hydrolysate is freed, as far as may be possible, from HCl by concentration *in vacuo* to a thick syrup. It is then taken up in water, neutralized to Congo-red with 40 % NaOH, decolorized with norite, filtered, and diluted to approximately its original volume. If its estimated concentration of arginine is then much greater than 1 % (as would be the case with a protamine hydrolysate) it may with advantage be diluted even further. The solution is now heated to the point of boiling, and treated with 20 % flavianic acid. The quantity of this to be used is calculated at the rate of 20 ml. for each g. of expected arginine. The mixture having been cooled, first to room temperature and then for at least 24 hr. in the ice-chest, the flavianate is collected under suction on a large fritted-glass funnel, washed, suspended in several vol. of water and brought into solution with the minimum amount of NH_3. The solution is diluted until it contains approximately 3 % of arginine, and is then digested with arginase and urease. The quantities of these are calculated so as to supply 1000 units of arginase and 1·4 ml. of urease solution for each g. of arginine believed to be present. Digestion is continued, in the presence of toluene, for 4 days at 37°.

With or without a preliminary vacuum distillation (see Section B) the strongly ammoniacal digest is neutralized with 5 *N* H_2SO_4 and boiled. Additional acid is added sufficient to ensure the exact coagulation of the proteins. The cooled mixture is treated with an adequate excess of saturated baryta water, and filtered. The precipitate is washed with dilute baryta. Filtrate and washings are combined, and freed from NH_3 by vacuum distillation just as in the preparation from arginine.

The further treatment of the material depends upon whether it is decided to isolate the ornithine as sulphate or as hydrochloride.

(*a*) *Ornithine monohydrochloride*. To obtain the hydrochloride the solution left in the distilling flask is filtered (if necessary), exactly freed from Ba with H_2SO_4, shaken with enough norite to remove residual flavianic acid, filtered, neutralized to litmus with HCl, and concentrated *in vacuo* to about 100 ml. The colour which usually develops during this concentration is removed by boiling with norite, and the filtered solution is evaporated to dryness. The crude hydrochloride is finally recrystallized from 75 % alcohol in the manner described already in Section B.

(*b*) *Ornithine monosulphate*. To obtain the sulphate the solution is neutralized exactly to litmus with H_2SO_4, a step which incidentally frees it from Ba. It is

then decolorized, filtered, concentrated, again decolorized—all in the same way as with the hydrochloride—and finally evaporated on the water bath, not to dryness, but only until crystallization begins. When it has cooled, it is stirred vigorously with successive small additions of absolute alcohol. This brings about the separation of more sulphate, at first in the form of milky globules, presently coalescing into a heavy oil. As the operation is continued, the oil gradually solidifies, the crystals get harder and harder, and finally the whole is converted into a heavy crystalline powder. More alcohol is added, and the mixture is left overnight in the ice-box. The alcohol is then poured off, and the crystals are washed, by stirring and decantation, 3 times with cold 95 % alcohol. The crystals are allowed to dry in the air, being stirred frequently the while, in order to prevent them from forming heavy crusts or sticking to the sides of the dish. They are then dried further in the desiccator and finally at 110°.

It will be gathered from this prescription that the physical properties of the sulphate differ from those of the hydrochloride. Kossel & Weiss [1909] describe an optically inactive ornithine sulphate which can be readily recrystallized from hot 60 % alcohol. The enzymically prepared sulphate, which is dextrorotatory, cannot be conveniently recrystallized in this way. Heated with either 60 or 75 % alcohol it yields for the most part only an oil. Recrystallization from 85 % alcohol is possible, but requires an impracticably large volume of solvent. Collected and dried on a filter in the usual way, the crystals form a hard cement-like cake, which can hardly be detached or broken up even with a steel spatula. By the methods of crystallization, washing and drying described above one escapes these several inconveniences.

The preparation of the sulphate involves fewer and simpler manipulations than that of the hydrochloride, and its yield is at least equally good. Its sole disadvantage is that the product may contain a rather high proportion—up to 3·6 % of ash. This consists mainly of calcium sulphate. It can be eliminated by repeated recrystallization from water, but the ornithine salt itself is so soluble, that purification by such means would be highly unprofitable.

The methods described have been applied with success to gelatin and to protamine, and Table I gives the data of three experiments with these materials. Two (with gelatin) exemplify the preparation of ornithine sulphate, the third (with protamine) that of the hydrochloride. The gelatin used was of the same brand as that employed in Part I for the preparation of arginine, so that 100 g. (air-dry) were capable of yielding 7·48 g. of ornithine monohydrochloride or 8·04 g. of ornithine monosulphate. The protamine was derived (as a sulphate) from an unidentified species of Pacific Coast salmon (*Oncorhynchus*). Its degree of purity was unknown. Analysis showed that it contained 11·9 % water, 18·5 % H_2SO_4 and 19·9 % N. On ignition it left 5·54 % ash, largely sulphate. The arginase method indicated that arginine accounted for 77·2 % of the total N—much less than the 89·3 % found by Waldschmidt-Leitz *et al.* [1931] for purified salmine. According to these data 100 g. of the air-dry sulphate contained 47·75 g. of potential arginine. The equivalent amount of ornithine monohydrochloride is 46·2 g. The high arginine content of protamine makes it a particularly remunerative starting material for the preparation of ornithine.

On a review of the figures in Table I it may be seen that, whether one starts from arginine itself or from protein, the enzymic method is capable of yielding from 80 to 90 % of the theoretical amount of ornithine. With respect to nitrogen content the product, whether in the form of sulphate or of hydrochloride, shows a high degree of purity, and only the sulphate contains more than a trace of inorganic impurity.

Table I

	Arginine hydrochloride		Gelatin		Protamine sulphate
Weight taken, air-dry (g.)	—		200		50
Weight taken, ash- and water-free (g.)	10·00		175·6		41·3
Arginine content (g.)	8·27		15·5		23·9
Ornithine monohydrochloride corresponding (g.)	8·00		—		23·1
Ornithine monosulphate corresponding (g.)	—		16·1		—
	(1)	(2)	(1)	(2)	
Ornithine monohydrochloride recovered (g.)	6·00	7·17	—	—	18·7
Ornithine monosulphate* recovered (g.)	—	—	14·4	14·0	—
Percentage yield*	75	90	90	87	81
Ash, % of product	0·28	0·10	3·63	3·38	0·04
Nitrogen,* % of product	16·42	16·48	15·47	15·20	16·52
Nitrogen, % of product, theoretical	16·62		15·47		16·62

* On an ash-free basis.

Determinations of specific rotatory power were made (1) on the monosulphate No. 1 from gelatin, (2) on the monohydrochloride from protamine, (3) on the dihydrochloride, prepared by adding the calculated amount of HCl to a solution of the monohydrochloride. The details and results of the measurements made are given in Table II. It should be stated that the first measurement on the monohydrochloride was made before, the others after, a single recrystallization from alcohol. This recrystallization, it will be seen, did not increase the specific rotation. The first and second measurements reported on the sulphate were made after, respectively, one and two recrystallizations from water. The product of the first recrystallization still retained 0·90 % ash; that of the second had only 0·16 %. The specific rotations of the two are not significantly different. The original sulphate, with 3·6 % ash, gave a value rather lower than that shown in Table II. Whether this should be attributed to a racemic admixture or to other impurities is uncertain.

Table II. *Specific rotations of ornithine salts*

					$[\alpha]_D$ calculated	
Salt of ornithine	t	l	c	α	For the salt	For ornithine
Monohydrochloride	23°	2·2	22·37	+4·98°	+10·13° } +10·08°	+12·85°
,,	23	2·2	21·99	+4·85	+10·03 }	
,,	23	2·2	5·496	+1·33	+11·00 } +11·0	+14·0
,,	25	2·2	5·693	+1·37	+10·94 }	
Dihydrochloride	23	2·2	13·37	+5·11	+17·36	+26·96
,,	25	2·2	10·60	+4·01	+17·20	+26·71
,,	23	2·2	5·300	+1·95	+16·7	+25·9
Monosulphate	22	2·2	6·070*	+1·15	+ 8·61 } + 8·7	+11·9
,,	22	2·2	5·864*	+1·13	+ 8·76 }	

* On an ash-free basis

As far as the mono-salts of ornithine are concerned, the only previous determinations of optical activity are that of Vickery & Cook [1931], who found for the enzymically prepared monosulphate $[\alpha]_D^{25°} = +8·4°$, and that of Dirr & Späth [1935], who give for the monohydrochloride $[\alpha]_D^{20°} = +12·12$. For the first of these salts the values here exhibited are appreciably higher, for the second quite definitely lower. The discrepancies may be more apparent than real, for neither Dirr & Späth nor Vickery & Cook have recorded concentrations or other relevant details. In any case the lower values now assigned to the monohydrochloride are supported by the results for the dihydrochloride, into which it was

converted. For this salt there are on record three earlier determinations—that of Schulze & Winterstein [1901], who found $[\alpha]_D = +16{\cdot}8°$ ($c = 5{\cdot}06$; $t = 10°$), that of Kossel & Dakin [1904], who give $+16{\cdot}6°$ (no details), and that of Bergmann & Zervas [1926], who give $+16{\cdot}5°$ ($c = 4{\cdot}72$; $t = 19°$). With these figures the present value $+16{\cdot}7°$ (for $c = 5{\cdot}30$ and $t = 23°$) is in satisfactory agreement. As far, therefore, as the data enable one to judge, the enzymically prepared material was pure $l(+)$-ornithine hydrochloride.

The specific rotatory power of free ornithine is given by Vickery & Cook (1931) as $+11{\cdot}5°$. From the figures in Table II it may be seen that the rotation increases as the base combines with increasing proportions of acid, and that the effect of HCl in this respect is greater than that of H_2SO_4. It is further evident that both for the mono- and the di-hydrochloride specific rotatory power varies more than a little with concentration. In all these respects the behaviour of ornithine shows, quantitatively as well as qualitatively, a striking resemblance to that of arginine [Gulewitsch, 1899].

Summary

Methods are described which exemplify (1) the use of "trypsin" in the preparation of arginine (as hydrochloride) from protein, and (2) the combined use of arginase and urease in the preparation of ornithine (as hydrochloride or sulphate) from arginine or from protein. The methods for ornithine give yields up to 90 % of the theoretical. The products are shown to possess a high degree of optical purity. The specific rotatory power of $l(+)$-ornithine monosulphate (in 6 % concentration) is $+8{\cdot}7°$, while that of the monohydrochloride varies from $+10{\cdot}08°$ (at 22 %) to $+11{\cdot}0°$ (at 5·5 %), and that of the dihydrochloride from $+17{\cdot}36°$ (at 13 %) to $+16{\cdot}7°$ (at 5 %).

In the development of the method for arginine the writer had the technical assistance of Mr C. H. Downs, and at all points of the investigation that of Mr C. E. Downs. The polarimetric observations were made by Mr A. G. Gornall. The protamine used was furnished by the Connaught Laboratories of the University of Toronto. To all these helpers the writer gratefully acknowledges his indebtedness.

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V. THE MANIFOLD ACTIVITY OF TESTOSTERONE DIPROPIONATE AS COMPARED WITH THAT OF TESTOSTERONE PROPIONATE IN GONADECTOMIZED RATS

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Enol esters of male sex hormones were first prepared by Ruzicka & Fischer [1936]. Miescher *et al.* [1937] have prepared several new members of this group, and have studied the effects of some of the most important testosterone compounds on capon's comb growth and on the weight of the seminal vesicles and prostate of rats. In a 10-day test the enol esters of testosterone increased the weight of the seminal vesicles less than did testosterone propionate, but with some of the diesters, in particular the dipropionate, the effect lasted much longer.

From the clinical point of view, preparations with more prolonged activity have evident advantages. Therefore the activity of one of the most interesting of the enol esters (according to Miescher *et al.* [1937]), namely, testosterone dipropionate, on weight and histological structure of the organs has been studied in this paper and compared with that of the propionate which is at present used almost exclusively in clinical practice. Testosterone propionate has already been studied by us [Korenchevsky, 1937, 1; Korenchevsky & Dennison, 1937; Korenchevsky *et al.* 1937; Korenchevsky *et al.* 1937, 1 and 2; Korenchevsky & Hall, 1937].

Technique

The pure crystalline hormones were supplied by Messrs Ciba Ltd., and were dissolved in sesame oil. Each dose was contained in 0·2 ml. of oil, and was injected daily into male or female rats, gonadectomized at the age of 22–24 days, i.e. before sexual maturity. The injections were continued in males for 23 days and in females for 21 days. The injections into the rats of groups "+10 days rest" (see Tables I and III) were commenced 10 days earlier than in the other groups, and these animals were not injected for the last 10 days before killing. In all the other groups the rats were killed on the day following the last injection. In this way the final age of all the rats in any one litter was the same.

The final age of the *female* rats was about the same in all groups (average 69 days). A large number of litters of *male* rats was used for the experiments, the final age varying in the different litters from 74 to 106 days.

Since in castrated control rats the average weights of some of the organs vary significantly according to age, for the sake of a more accurate comparison two average weights are given for these organs in the group "control castrated rats" (Table I, col. II): (1) upper figure—average age 96 days, (2) lower figure—average age 78 days. For the other organs of rats in this group, the general average age was 88 days. The average age of the normal control rats was 95 days, there being no normal controls to the younger rats.

Table I. *Effect on actual weights of organs of castrated rats of testosterone dipropionate alone or in combination with oestradiol dipropionate*

	I	II	III	IV	V	VI	VII	VIII	IX	X
			Castrated rats injected with							
	Control rats injected with oil		Testosterone dipropionate							
Organs	Normal	Castrated	50 μg.	150 μg.	150 μg. + 10 days' rest	500 μg.	500 μg. + 10 days' rest	1500 μg.	Oestradiol dipropionate 6 μg.	Oestradiol dipropionate 6 μg. + testosterone dipropionate 150 μg.
Seminal vesicles (mg.)	952	12	163	528	333	1026	1022	1197	51	601
Prostate (mg.)	853	65	330	624	557	963	1135	1028	86	677
Penis (mg.)	348	81	218	282	280	327	356	316	71	271
Preputial glands (mg.)	135	37	95	117	119	140	119	128	43	106
Adrenals (mg.)	58	74	71	55	54	58	52	56	59	78
Hypophysis (mg.)	9·5	10·6	12·7	11·6	11·3	12·3	11·3	9·3	20·7	21·8
Thymus (mg.)	349	472	530	477	490	188	228	142	309	242
Liver (g.)	12·37	$\frac{10·48}{9·22}$	11·74	13·06	12·24	11·24	12·07	10·05	9·16	9·43
Kidneys (g.)	2·32	$\frac{1·83}{1·61}$	1·96	2·29	2·06	2·32	2·23	1·92	1·85	2·10
Heart (mg.)	1047	$\frac{920}{747}$	984	966	956	1052	1018	855	756	896
Retroperitoneal fat (g.)	13·0	11·1	10·2	11·9	10·1	11·2	13·5	8·0	8·4	11·6
Final body weight (g.)	342	$\frac{310}{275}$	313	310	305	329	336	254	245	290
Gain in body weight (g.)	116	$\frac{100}{132}$	155	160	148	104	99	115	77	86
No. of rats in group	6	7	5	8	8	4	4	4	4	4
Final age (days)	95	$\frac{96}{78}$	78	80	80	104	99	77	96	82

For economy of space, the data per unit of body weight have not been tabulated, but will where necessary be referred to in the text.

A detailed account of the results of histological investigation will be given elsewhere, only some preliminary statements being included here.

Other details of the experimental technique can be found in our previous papers.

Castrated male rats

The experiments were performed on 59 rats, divided into 11 groups as shown in Table I (groups I–X) and Table II (group XI). Groups XII and XIII in Table II contain additional data taken from our previous experiments on 8 rats [Korenchevsky *et al.* 1937, Table I].

Table II. *Effect on actual weights of organs of castrated rats of testosterone propionate in doses comparable with those of testosterone dipropionate in Table I*

	XI	XII	XIII
	Rats injected with testosterone propionate		
Organs	50 μg.	500 μg.	500 μg. + 10 days
Seminal vesicles (mg.)	417	1228	284
Prostate (mg.)	507	1248	614
Penis (mg.)	256	301	276
Preputial glands (mg.)	103	154	118
Adrenals (mg.)	60	59	59
Hypophysis (mg.)	11·4	12·5	13·3
Thymus (mg.)	502	219	291
Liver (g.)	10·49	12·1	11·52
Kidneys (g.)	1·79	2·03	1·84
Heart (mg.)	864	932	927
Retroperitoneal fat (g.)	9·8	12·2	13·1
Final body weight (g.)	288	316	318
Gain in body weight (g.)	144	122	116
No. of rats in group	5	4	4
Final age	74	89	89

Sex organs

Injections of 500 μg. of testosterone dipropionate daily (Table I, group VI) cause complete return to normal size, weight and histological structure of the seminal vesicles, prostate, penis and preputial glands, and this effect remains unchanged, or is even increased, 10 days after the last injection (group VII). Injections of 150 μg. are unable to restore the size and weight of these glands completely to normal, and the restorative effect obtained in the seminal vesicles, and less so in the prostate, begins to decrease during the period without injections, the weights of the penis and preputial glands remaining unchanged. Increasing the daily dose to 1500 μg. (group VIII) does not produce a significantly greater effect than that obtained with 500 μg. (groups VI and VII). Simultaneous injections with oestradiol dipropionate (group X) show only a very weak co-operative effect (as judged by weight) on the seminal vesicles and prostate.

Comparison with testosterone propionate. While with the small dose of 50 μg. (Table I, group III and Table II, group XI) the activity of the dipropionate is considerably less than that of the propionate, this difference is much less pronounced with the medium dose of 500 μg. (Table I, group VI and Table II, group XII).

Supernormal seminal vesicles (1441 mg. average) are produced by 1400 μg. of the propionate [Korenchevsky *et al.* 1937, Table I, col. IV] but not by 1500 μg. of the dipropionate (this paper, Table I, group VIII). Thus the dipropionate is weaker than the propionate, although the difference is considerable only with the small doses.

A more important difference is the fact that the effect of 500 μg. of the dipropionate is maintained or even increased 10 days after the injections have ceased (Table I, group VII), and even with 150 μg. there is a considerable decrease only in the seminal vesicles (group V) while the effect of all doses of the propionate is very considerably decreased by the tenth day [Korenchevsky *et al.* 1937; see also Table II of this paper, groups XII and XIII].

The dipropionate appears to have a slightly better co-operative activity with oestradiol, since with the propionate [Korenchevsky & Dennison, 1937] an effect was recorded only on the seminal vesicles.

Other organs

The changes produced by the dipropionate on the weights of other organs are similar to, or even greater than, those caused by the propionate. The effects are maintained for at least 10 days with both hormones.

Adrenals. The hypertrophied "castration" adrenals return to normal size and weight (except with the lowest dose) this restoration being explained by a decrease in the width of the cortex. Only the largest dose of 1500 μg. causes considerable depletion of lipoid granules in the cortex.

Thymus. Involution, delayed in castrated animals, returns to or exceeds the normal level (as shown by weight and histologically) with medium and large doses of the hormone.

Liver, kidney and heart. The weights (actual and per unit body weight) of these organs, decreased after castration, return to or towards normal. Since histologically nothing abnormal (liver, heart) or a slight increase of some tubules or their cells (kidneys) was found, the enlargement obtained should be considered as a stimulation of these organs by the injections.

Hypophysis. It is difficult to decide how much significance should be attached to the slight enlargement of the hypophysis observed in most rats injected with all but the largest dose of the hormone. A certain amount of variation was present in all groups and the results obtained with the largest dose were rather indefinite. Oestradiol dipropionate, as usual, produced hyperplasia, which the simultaneous injection of testosterone dipropionate, in the dose used, did not prevent (Table I, groups IX and X).

Thyroids, parathyroids, pancreas and spleen did not show any changes.

Fat deposition and gain in body weight. There appeared to be a more pronounced gain in body weight with small doses of the dipropionate (groups III–V), but no definite changes in fat deposition, so that the increase cannot be explained by accumulation of fat in the body. 500 μg. produced no definite change (groups VI and VII), while with 1500 μg. (group VIII) both fat deposition and also gain in body weight were slightly decreased. The dipropionate, therefore, appears to have some effect on metabolism.

Simultaneous injections of oestradiol dipropionate (group X) neutralize the effects of the male hormone on the adrenals and also prevent more or less its stimulating activity on liver, heart and gain in body weight, but not the enlargement of the kidney (groups IX and X). Co-operative activity of the two hor-

mones is shown in the acceleration of the involution of the thymus. Similar results have been obtained previously after the addition of oestradiol to testosterone propionate [Korenchevsky & Dennison, 1937].

Ovariectomized females

Experiments were performed on 36 rats belonging to 8 litters and divided into 8 groups as shown in Table III.

Table III. *Effect on actual weights of organs of spayed female rats of testosterone dipropionate alone or in combination with oestradiol dipropionate (and compared with the effect of testosterone propionate)*

	I	II	III	IV	V	VI	VII	VIII
			Spayed rats injected with					
	Control rats		Testosterone dipropionate					
	Normal	Spayed	150 μg.	150 μg. + 10 days rest	1500 μg.	Oestradiol dipropionate 6 μg.	Oestradiol dipropionate 6 μg. + testosterone dipropionate 150 μg.	Testosterone propionate 1500 μg.
Uterus (cervix + horns, mg.)	359	31	113	63	246	212	245	242
Cervix of uterus (mg.)	103	13	48	29	111	92	109	114
Vagina (with clitoris, mg.)	241	168	280	259	410	240	337	338*
Preputial glands (mg.)	97	66	126	95	176	66	109	255
Adrenals (mg.)	58	68	48	42	50	69	68	62
Hypophysis (mg.)	11·8	11·0	9·6	7·7	7·8	21·6	18·5	11·8
Thymus (mg.)	382	590	445	487	147	257	257	87
Liver (g.)	8·03	8·45	9·68	10·32	8·69	8·58	8·55	8·73
Kidneys (g.)	1·42	1·51	1·69	1·71	1·74	1·77	1·89	1·76
Heart (mg.)	665	708	765	750	744	689	681	764
Retroperitoneal fat (g.)	9	13	13	12	9	8	9	5
Final body weight (g.)	191	236	243	240	225	188	205	213
Gain in body weight (g.)	70	95	108	95	82	56	69	53
No. of rats in the group	9	8	4	3	3	3	4	2

* In group VIII the vaginal weight is given without clitoris.

The data in group V (testosterone dipropionate) are compared with those given in group VIII, from rats injected with testosterone propionate (1500 μg.). The testosterone compounds were injected daily and the oestradiol dipropionate three times per week in the doses given in the table.

Sex organs. The restorative effect on the sex organs of the spayed rats was very pronounced, and similar to that of the propionate (compare groups V and VIII). The weight of the vagina after injections of both 150 and 1500 μg., especially the latter, exceeded that of the normal control, but complete recovery of the uterus was not obtained even with the large dose. The duration of the effect of the injections was not studied, except in the case of the small dose (150 μg., group IV), when the effect, especially on the uterus, decreased considerably during the 10 days after the injections were discontinued.

There was definite co-operative activity with oestradiol dipropionate on the uterus and especially on the vagina, but not on the preputial glands (group VII).

Histological changes in the sex organs were similar in character but not in degree to those obtained with testosterone propionate [Korenchevsky & Hall,

1937]. With 150 μg. a dioestrus uterus and metaplasia of the vaginal epithelium into small mucous cells were obtained, but with 1500 μg. the progestational changes both in the uterus and vagina approached much more closely than with the propionate to those seen during pregnancy. Thus the dipropionate possesses, even more strongly than the propionate, this remarkable property of producing progestational changes in the sex organs of ovariectomized animals, without any previous sensitization with oestrogens. The histological changes produced by the injections were maintained during the 10 days' "rest" even less than were the weight changes: the uterus became atrophic, and the mucous metaplasia in the vagina partly, or in some rats entirely, disappeared.

Co-operative and antagonistic activities of testosterone dipropionate and oestradiol dipropionate

These two hormones when injected simultaneously show both co-operative and antagonistic activities.

Miescher *et al.* [1938] have shown that oestradiol dipropionate has a greater and more prolonged activity than pure oestradiol. This is also shown in the present experiments, when 6 μg. of the dipropionate produced about the same effect by weight and histologically as 30 μg. of pure oestradiol [Korenchevsky & Hall, 1937]. In the uterus, 6 μg. of the dipropionate produced the same high cylindrical epithelium, transformed in places into squamous epithelium (metaplasia), as [Korenchevsky & Hall, 1938] that with 30 μg. of oestradiol. This metaplastic epithelial change is important as indicating the first step in the "carcinogenic" activity of oestrogens.

Simultaneous injections of testosterone dipropionate and oestradiol dipropionate gave a picture typical of the female hormone. Thus oestradiol dipropionate is *antagonistic* to and overrules the activity of testosterone dipropionate. It is important that the doses used of testosterone dipropionate did not prevent the metaplastic changes produced by oestrogens in the uterus, while these changes were prevented by progesterone [Korenchevsky & Hall, 1938].

A *co-operative* activity of the dipropionates of testosterone and oestradiol was shown in a better recovery in weight and size of the uterus and vagina.

Effect on female prostate and clitoris, and classification of the hormone. The injections caused a great development of the female prostate and clitoris, similar to that described previously for the other male hormones [Korenchevsky, 1937, 2; Hall, 1938].

The female prostate, unlike the uterus and vagina, did not regress during the 10 days after the cessation of the injections (group IV). Moreover, 1500 μg. and even 500 μg. of testosterone dipropionate overstimulate the male organs, but 1500 μg. are unable to restore the uterus to normal. These facts place testosterone dipropionate (using the classification suggested by Korenchevsky [1937, 1]) in the group of "bisexual hormones possessing chiefly male properties."

Other organs, fat deposition and gain in body weight

Thymus and adrenals. Testosterone dipropionate had a similar effect on the weights and histological structure of these organs in females as in males.

Liver, kidneys, heart, fat deposition and gain in body weight. The general effect on these organs appears to be similar in character to that in males, but weaker in degree and less constant. More numerous experiments, and especially of longer duration, are needed in order to come to a definite conclusion. No pathological changes were found histologically in the liver and kidneys.

The similarity of these changes in the two sexes is rather remarkable, as the effects of gonadectomy on most of the organs investigated were different in males from those in females. This will be discussed in more detail elsewhere.

When injected simultaneously (group VII) oestradiol dipropionate was found to neutralize more or less completely the activity of testosterone dipropionate on adrenals, hypophysis (increased weight), liver, heart, fat deposition and gain in body weight (decrease), while stimulation of kidneys was evident both in actual weight and per unit of body weight.

Summary

1. In gonadectomized male and female rats the manifold effects of testosterone dipropionate are in general similar to those of testosterone propionate, with, however, some important differences.

On male sex organs

2. The effect of small doses of the dipropionate on these organs is weaker (e.g. on the prostate, by about 1·5 times) than that of similar doses of the propionate. With larger doses this difference is much less.

3. In contrast to the propionate, however, the effect of the dipropionate on the male sex organs is maintained (completely with 500 μg., partially with 150 μg.) for at least 10 days after the last injection.

On female sex organs

4. Testosterone dipropionate alone, without previous stimulation with oestrogens, can produce progestational changes in the uterus and vagina, much more similar to those found during pregnancy than the changes produced by the propionate.

5. After the cessation of the injections (small doses) the development of the vagina is maintained better than that of the uterus.

6. Testosterone dipropionate has very pronounced bisexual properties (including strong stimulation of the female prostate and clitoris), but, from a comparison of its "male" and "female" activities, it can be classified as a "bisexual hormone possessing chiefly male properties".

7. Testosterone dipropionate, in the dose used, did not prevent the metaplastic changes produced in the uterine epithelium by oestradiol dipropionate.

Non-sexual organs of both sexes

8. Testosterone dipropionate influenced the thymus, adrenals, liver, kidneys, heart, fat deposition and gain in body weight in both sexes, but in most cases, with the doses used, the effects in females were less pronounced and less constant than in males.

9. Testosterone dipropionate and oestradiol dipropionate show both co-operative and antagonistic activities on sexual and non-sexual organs.

10. The experimental evidence obtained indicates a possible advantage of testosterone dipropionate over the propionate for clinical use.

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VI. PREPARATION OF α-AMINO-ACIDS THROUGH α-OXIMINO-ESTERS

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Amino-acids free from biologically important impurities which may accompany those prepared from natural sources have been required in this department for the study of bacterial nutrition. Synthesis of simple amino-acids presents little difficulty by the usual routes, but serviceable methods are still required in certain cases, particularly if ring closure of intermediate derivatives is apt to occur. We have therefore extended to new compounds the ethyl acetoacetate synthesis of Bouveault & Locquin [1904; 1906] and Schmidt & Widman [1909] via α-oximino-esters, since this method has also the merit of accessible starting materials. Difficulties in the earlier work have been minimized by using the convenient catalytic reduction of oximino-esters applied by Harington & Randall [1931] to hydroxyglutamic acid. This reduction may have considerable biological interest, since oximinosuccinic acid has been isolated by Virtanen [1938] as an intermediary in the production of aspartic acid during nitrogen assimilation by legume bacteria, of which process it probably constitutes the first stage.

Glutamic acid has been successfully synthesized in an overall yield of 39 % as compared with 18·5 % in the best method hitherto available from ethyl malonate [Dunn *et al.* 1931], and the even smaller yields from other sources [cf. Dunn *et al.* 1931]. Hydroxyproline has been prepared, but the yield was not superior to that of Leuchs [1905]. The synthesis of threonine has been attempted, but was discontinued following the publication by Adkins & Reeve [1938] of a satisfactory synthesis of that acid by an analogous method. Other preparations have been deferred in accordance with the needs of our work.

Experimental

Glutamic acid

Diethyl-α-acetylglutarate was prepared by the following modification of the method of Clemo & Welch [1928]. To a cooled solution of sodium (4·03 g.; 0·175 mol.) in absolute alcohol (53 ml.) ethyl acetoacetate (44 ml.; 0·35 mol.) was gradually added, followed by ethyl β-chloropropionate (21 ml.; 0·175 mol.). The mixture was kept 3 hr. at room temperature and refluxed for 2 hr.; during the last ½ hr. the *p*H of an aqueous solution of a drop of the mixture remained at 9. Alcohol was removed at 100°, NaCl which separated during the process dissolved in the minimum quantity of water and the aqueous solution extracted with ether. The ethereal extracts were washed with very dilute HCl, dried with $CaCl_2$, evaporated and the residue distilled. Excess ethyl acetoacetate distilled first, and the glutaric ester (31·8 g.; 79 % theory) at 146–150°/12 mm.

Diethyl-α-oximinoglutarate was prepared from the acetyl compound according to Wislicenus & Grutzner [1909].

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Glutamic acid. The freshly prepared oximino-ester (16 g.; 0·075 mol.), platinum oxide (0·4 g.; Adams *et al.* [1932]) and anhydrous Na_2SO_4 (15 g.) in acetic acid (60 ml.; redistilled) were shaken in hydrogen at a little over atmospheric pressure until the theoretical uptake had taken place (3 days). The solution was filtered, water added, the further precipitate of catalyst filtered off and the solution taken to dryness. The amino-ester remaining was immediately hydrolysed by refluxing with 5 *M* aqueous HCl (50 ml.), the product evaporated to 25 ml. under reduced pressure, cooled in ice and saturated with HCl; the glutamic acid hydrochloride was collected after keeping for 12 hr. at 0°. Yield 9·0 g. A further crop of about 1 g. was obtained from mother liquors.

The salt was dissolved in water, freshly distilled aniline (20 g.) added, the solution warmed and ethyl alcohol added till crystallization began. The glutamic acid was crystallized from water and washed with alcohol; M.P. 197°. Yield 7·8 g., i.e. 39 % of the theoretical from the β-chloropropionic ester used. (Found: amino-N, 9·5 %; $C_5H_9O_4N$ requires 9·5 %. *Hydrochloride*, M.P. 198°; *picrolonate*: found, amino-N 3·4 %; $C_5H_9O_4N.C_{10}H_8O_5N_4$ requires 3·4 %.)

Hydroxyproline

α-Oximino-δ-chloro-γ-valerolactone. α-Acetyl-δ-chloro-γ-valerolactone [Leuchs, 1911]; B.P. 135–137°/1 mm.) (17·6 g.; 0·1 mol.) was added with vigorous stirring to conc. H_2SO_4 (20 ml.) cooled in ice-salt. Nitrosylsulphuric acid (12·7 g.; 0·1 mol.) in conc. H_2SO_4 (12 ml.) was added during ½ hr., the whole poured on to crushed ice, the product extracted with ether, the ethereal solution washed quickly with water followed by aqueous Na_2CO_3, dried and evaporated. The oxime remained as an oil, decomposing violently at 170° on attempted distillation but separating from benzene as pale yellow crystals. Yield, 11 g. (67 %) M.P. 118°. (Found: N, 8·6 %; $C_5H_6O_3NCl$ requires N, 8·6 %.)

α-Amino-δ-chloro-γ-valerolactone salts. The oxime (8·2 g.; 0·05 mol.), platinum oxide (0·4 g.), anhydrous Na_2SO_4 (5 g.) and glacial acetic acid (50 ml.) were shaken in hydrogen for 3 days; the solution was filtered and evaporated and the residue rubbed with alcohol. The amine acetate separated and was recrystallized from aqueous alcohol, yielding 2·9 g. (0·014 mol.) of colourless prisms M.P. 177°. (Found: amino-N, 7·0 %; $C_5H_8O_2NCl.C_2H_4O_2$ requires 6·7 %.) A similar yield of hydrochloride was obtained on reducing the oxime in alcoholic HCl.

Hydroxyproline. α-Amino-δ-chloro-γ-valerolactone acetate (4 g.) with saturated aqueous ammonia (80 ml.) was warmed at 30°. Titration of small portions indicated the halogen to be completely eliminated as Cl^- after 2 hr. Hydroxyprolines *a* and *b* were isolated according to Leuchs [1905]. Yields: *a*, 0·1 g., M.P. (d.) 254°. (Found: N, 10·4 %; $C_5H_9O_3N$ requires 10·7 %); *b*, 0·7 g., M.P. (d.) 244°. (Found: N, 10·5 %.)

Threonine

Reduction of ethyl-α-oximinoacetoacetate. Reduction and hydrolysis under the conditions used by Harington & Randall [1931] yielded only α-aminobutyric acid; yield, 80 %. (Found: N, 13·4 %; $C_4H_9O_2N$ requires 13·6 %.) It was not found possible to arrest the reduction at the hydroxy-acid stage.

Ethyl-α-amino-β-ketobutyrate hydrochloride. This intermediary in the above reduction was isolated in order to attempt its reduction by other methods. Ethyl isonitrosoacetoacetate (8·0 g.) palladium charcoal (3·7 g.) and alcoholic HCl (50 ml. 2 *N*) were shaken in hydrogen. Theoretical uptake occurred in 5 hr. The solution was filtered and evaporated under reduced pressure to a few ml., when the hydrochloride separated in white prisms. It was recrystallized from

alcohol with the addition of light petroleum. Yield, 62%, M.P. 125°. (Found: N, 7·85%; $C_6H_{11}O_3N.HCl$ requires 7·7%.) The salt exhibited the properties described by Gabriel & Posner [1894] and by Cherchez & Cherbuliez [1931] but was obtained in better yield and melted much higher than described by those authors. Attempted further reduction yielded only α-aminobutyric acid or diethyl-2:5-dimethylpyrazine-3:6-dicarboxylate.

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VII. ON THE RELATIONSHIP BETWEEN THE SULPHUR CONTENT AND THE ANTICOAGULANT ACTIVITY OF HEPARIN PREPARATIONS

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About 20 years elapsed between the discovery of heparin by Howell in 1916 and its chemical identification. Howell [1928] reported on preparations which must have been reasonably pure and showed a positive naphthoresorcinol reaction, indicating the presence of a hexuronic acid. Fischer & Schmitz [1933], as well as Charles & Scott [1933], also worked with preparations of a high degree of purity, considered by them to be crystalline or semi-crystalline. Fischer & Schmitz [1933] claimed that they had succeeded in preparing a crystalline brucine salt of heparin. The elementary composition of this heparin indicated that it contained neither sulphur nor nitrogen. Their statement was unjustified, however, for on ignition the samples yielded a small amount of ash and were thus not homogeneous but a mixture of brucine and alkali salts.

Heparin was found by Jorpes [1935] to be a polysulphuric ester of chondroitin or some closely related substance. When the amino-sugar was identified as glucosamine [Jorpes & Bergström, 1936] heparin seemed to be a mucoitin polysulphuric acid. The occurrence of sulphur, accidentally observed by Howell already in 1928, was later denied [Fischer & Lipmann, 1935; Schmitz, 1935], but sulphur was soon afterwards recognized as an essential component of the active substance [Charles & Scott, 1936].

A new contribution to the discussion was made by these latter authors, Charles & Scott, in that they claimed that they had succeeded in isolating a crystalline barium salt of heparin. This salt would thus have to be regarded as the first chemically pure preparation of the anticoagulant. The free acid, derived from the barium salt as well as from an amorphous benzidine salt, was believed to have the composition $C_{25}H_{65}O_{50}N_2S_5$, with 11·5 % S. This statement has led to some confusion concerning the chemical nature of heparin, for all the evidence previously obtained indicated that heparin was not a definite chemical compound but a mixture of different polysulphuric esters, i.e. a polysaccharide (mucoitin) which could be esterified with sulphuric acid to a varying degree and might contain from 20 to 40 % of sulphuric acid. With increasing S content there was a steady increase in the anticoagulant activity. The different esters, having different S contents, could easily be separated as brucine salts by fractionation in water. The organic skeleton appeared to be the same in all of them, hexuronic acid, hexosamine (glucosamine) and acetic acid together making up at least 90 %. All this evidence was available in 1935.

It is quite natural that in the meantime those who have reviewed the subject have considered this alleged crystalline product to be pure heparin. They have not paid much attention to the fact that the amorphous preparations can contain 20 % more S (up to 13·5 % in the free acid) than the crystalline product, which has 11·5 %. The more recent discussions have rather neglected the previously

established facts. Thus Charles & Scott [1936] did not feel convinced that heparin contained hexuronic acid and hexosamine, in spite of the fact that, by means of most reliable reactions for detecting sugars the Tollens-Lefèvre procedure and the pyrrole reaction with Ehrlich's reagent for amino-sugars, they had been shown to make up 90 % of the ash-free preparations, if acetic acid also is assumed to occur in heparin. They found a considerable part of the nitrogen to occur in NH_2 groups. The amino group of heparin, however, is monoacetylated, $—NH.OC.CH_3$, as everywhere in the amino-sugars in nature. Of course part of it can become de-acetylated during the process of preparation, which includes heating with acids as well as with alkali, just as occurs in the preparation of chondrosine (mucosine) by acid hydrolysis. This negative aspect was most recently clearly expressed by Best [1938], who claims that "tests do not indicate the presence of glucuronic acid, which some previous observers had thought was present". The situation is somewhat perplexing when biochemists of to-day are unable to trace a substance which constitutes 25–30 % of the preparation and which was discovered by a physiologist 10 years ago. However, this matter need not be further discussed. The quantitative analysis according to Tollens-Lefèvre is quite reliable. The titration of the free acid obtained by electrodialysis showed at an early stage that there was one carboxyl group to 2 or 3 sulphate groups. The titration curves also prove, as did the Van Slyke analysis, that the amino group is acetylated [Wilander, 1938].

In Charles & Scott's formula for the crystalline product there is one detail which immediately disproves its correctness. There are 2·5 atoms of S to each N atom. The same observation was made by Jorpes [1935] as the result of analysing the earliest samples of pure heparin available. It seemed improbable that heparin should contain 2·5 sulphate groups in each chondroitin (mucoitin) complex. A more plausible explanation was then obtained when it was found that the preparations could be divided by means of brucine into fractions having the composition of chondroitin (mucoitin) di- and tri-sulphuric acids and mixtures of these. With increasing S content there was, as mentioned, an increase in the anticoagulant activity. Additional analyses indicating the same thing were later published by Jorpes & Bergström [1937]. Heparin has therefore always been regarded by us as a mixture of mucoitin polysulphuric esters.

The question as to the homogeneity of the product of Charles & Scott, supposed to be crystalline, is consequently of great interest. It will also be of importance when the question arises as to what is to be an international standard of heparin. In order to check our results we have repeated our earlier experiments on a larger scale and have subjected the different fractions of heparin to a very careful S analysis and a thorough biological standardization. No corrections of our earlier views have been found necessary.

The fractionation of ordinary pure heparin by means of brucine salts

The heparin used was the commercial product supplied by Vitrum of Stockholm. It is a sodium salt prepared mainly in accordance with the Charles & Scott method and contains about 12 % moisture, 33 % ash, 2 % N and 9–10 % S. It is 8–10 times more active than the older commercial samples, its activity being fairly constant. This sodium salt is used in the intravenous treatment of thrombosis in man. The free acid was prepared by electrodialysis against parchment in an apparatus constructed by E. Hammarsten [see Ågren, 1934]. Either 25 g. of heparin dissolved in 200 ml. were electrodialysed for 48 hr. or smaller samples were treated for a shorter time. Electrodialysis was continued until no

alkali was traceable with litmus in the cathode liquor. The acid was neutralized with brucine and the brucine salt fractionated in water. One part of it was soluble in water. In separating the insoluble fraction a clear mother liquor was obtained only after repeated freezing and thawing. This fraction was dissolved in boiling water and again frozen out. By repeating this procedure a series of mother liquors was obtained and a final less soluble brucine salt. It was also possible to separate a small fraction which was insoluble even in a large volume of boiling water. This last fraction showed the highest S content and the greatest anticoagulant activity.

The various brucine salts were treated with a small excess of NaOH and the brucine was removed with chloroform. The solution was then neutralized with HCl against litmus and the heparin precipitated with 1·5 vol. acetone. The precipitate was collected the following day, dissolved in a small amount of water and reprecipitated with alcohol.

For S analysis the substance was fused with carbonate-nitrate in a porcelain crucible. The precipitate of $BaSO_4$ was collected on a Neubauer-Gooch platina-iridium filter, ignited, washed with HCl and dried. The error of the method is insignificant. On two occasions samples were sent to Dr Schoeller of Berlin for control analysis, the same figures then being obtained as by the ordinary method.

The biological standardization was performed on fresh ox blood as described by Jorpes [1935]. This method easily discloses differences of 20 % and usually differences as small as 10 % [see also Wilander, 1938]. At least 8 stands with 10 test tubes (5 standard and 5 unknown) were used for each standardization, for some of them 14 or 16 stands. The figures in Tables I–III ought to be correct to within 10–15 %.

Two preparations of 25 g. each were fractionated; the results are presented in Tables I and II. The experiment given in Table III is incomplete.

Table I. *Fractionation of 25 g. heparin as brucine salts. Yield of sodium salts after removal of the brucine*

Source of substance	Wt. air-dry substance g.	Anticoagulant activity % standard	S % of dry substance
1st mother liquor	3·37	60	7·42
2nd mother liquor	5·30	45–50	7·01
3rd mother liquor	5·25	100	10·97
Less soluble brucine salt	2·30	125	11·74
Insoluble brucine salt I	0·266	150–160	11·88
Insoluble brucine salt II	0·238	150	11·96
	16·72		

Table II. *Fractionation of 25 g. heparin of standard potency as brucine salts. Yield of sodium salts after removal of the brucine*

Source of substance	Wt. air-dry substance g.	Anticoagulant activity % standard	S % of dry substance
1st mother liquor	5·12	20–25	8·52
2nd mother liquor	6·52	75–80	9·58
3rd mother liquor	2·73	100–110	11·58
4th mother liquor	1·51	100	11·20
Less soluble brucine salt	0·61	140–150	12·13
Insoluble brucine salt I	0·32	130	11·72
Insoluble brucine salt II	0·09	150–160	12·26
	16·90		

Table III. *Fractionation of standard heparin as brucine salts. Yield of sodium salts from the most soluble fractions*

Source of substance	Anticoagulant activity % of standard	S % of dry substance
1st mother liquor	75	9·31
2nd mother liquor	80	10·41

Table IV. *Sulphur contents and anticoagulant activities of different heparin preparations*

S % of dry substance	Anticoagulant activity % of standard
7·01	45–50
7·42	60
8·52	20–25
9·31	80
9·58	75–80
10·41	80
10·97	100
11·20	100
11·58	100–110
11·74	125
11·72	130
11·88	150–160
11·96	150
12·13	140–150
12·26	150–160

It is evident from Table IV, where the samples are arranged in a series, that the anticoagulant activity increases with increasing S content. Only one sample (no. 3) out of 15 breaks the series, and that was obtained from the first mother liquor of this preparation. It is also apparent from the table that further additions of S cause a greater increase in the activity if introduced in the highly esterified samples, thus indicating that it is the abundance of ionic charges that makes the polysaccharide an anticoagulant. The bulk of evidence now available indicates that the activity is due to the ionic charge of heparin [Jorpes, 1938].

The question arises whether the preparations with lower S content consist of highly esterified heparin and impurities. It has already been shown that their organic skeleton is the same, i.e. mucoitin. The content of uronic acid is considerably higher in these samples than in those with high S and ash contents. The amino-sugar is also demonstrated to be the same, glucosamine. The original assumption that ordinary heparin is a mixture of different poly- (di- and tri-) sulphuric esters thus seems to be justified.

The properties of the heparin preparations are strongly influenced by the introduction of additional sulphuric acid groups. This makes their barium and brucine salts less soluble or insoluble in water. The salts of the samples with 7·0–7·42 % S behave like salts of chondroitin sulphuric acid, and they are not precipitated with barium chloride or barium hydroxide. On the other hand, the fractions with a content of S greater than 10·41 % give less soluble or insoluble barium and brucine salts. This excludes the possibility that the former fractions contain considerable amounts of the highly esterified stronger heparin. In fact, an attempt was made to fractionate one of these samples (7·01 % S) once more with brucine. After electrodialysis the solution was neutralized with

brucine. No insoluble brucine salt was precipitated. After cooling the concentrated solution, part of the brucine salt separated out and was removed in the centrifuge. From 5 g. containing 7·01 % S there were obtained 0·97 g. of a fraction containing 5·17 % S and 2·15 g. of a fraction containing 7·80 % S. The anticoagulant activities were 15 and 50 % of standard respectively. As could be expected, at least the first fractions shown in Table IV are not homogeneous, but they do not contain any considerable amount of highly esterified heparin.

Standard heparin

It will become necessary in the future to have a reference substance, a standard of heparin, as is already the case with other biological products used in medicine. A homogeneous crystalline product, if available, would be valuable for that purpose. However, the amorphous preparations of the pure liver heparin already have a very constant strength. For this reason Bergström *et al.* [1937] suggested that these should be used as standard. The same constant potency was found by Murray & Best [1938]. The potency of these liver preparations is sufficiently constant for practical use, varying by not more than 10–15 % in different preparations. This strength has been used by us as standard. We express the anticoagulant activity in mg. of this standard, considering this to be the most practical expression for that activity.

Recently at least six different units have been suggested by various workers, a tendency which would appear undesirable. Any method of assay may of course be applied in order to check the potency of a preparation against a standard, but the strength can hardly be expressed in a simpler way than in milligrammes of this standard. There is no reason for the use of smaller units, for in blood analysis as well as in therapy quantities of the order of milligrammes will be dealt with.

Conclusions

It is evident from Tables I–III that the anticoagulant activities of different heparin preparations obtained in a similar manner from the liver, vary with their S contents. Preparations of liver heparin containing from 7 to 12·26 % S show potencies varying from 45 to 160 % of the activity of the standard heparin from which they are derived. Pure liver heparin is a mixture of mucoitin polysulphuric esters which can be separated from each other as brucine salts. The starting material has a fairly constant strength and is used as heparin standard, the anticoagulant activity being expressed in milligrammes of this standard.

These preparations are sodium salts of heparin. The most potent samples, which contain about 10 % Na, contain about 13·5 % S in the free acid. For this and other reasons the alleged crystalline barium salt of heparin isolated by Charles & Scott in 1936, which has a content of S corresponding to about 11·5 % in the free acid, cannot be considered as the anticoagulant substance itself. Its homogeneous nature must be doubted. In any case, when this substance is discussed it must be kept in mind that it is merely a fraction taken out from the series of different polysulphuric esters of mucoitin which constitute heparin.

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VIII. A NEW TYPE OF MICRORESPIROMETER

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Introduction

Manometric methods are among the most useful and widely applied techniques in the field of biochemistry. The respirometers of Barcroft or Warburg are most commonly used, and the theory and practical manipulation of these and other types has been summarized by Dixon [1934].

These instruments are capable of measuring gas exchanges of not less than about 30 μl./hr., so that 5–50 mg. of tissue are normally used. However, there is a need for methods of measuring very much smaller gas changes, and several instruments capable of measuring O_2 uptakes of 1 μl./hr. or less have been described during the last decade. Stefanelli [1937] has described a miniature Barcroft-Warburg type of instrument. In others the respiration chamber is connected to a horizontal length of capillary tubing, the movements of a drop of fluid in the latter indicating the changes in volume [Fenn, 1927; 1928; Jackson *et al.* 1930; Schmitt, 1932; 1936; Gerard & Hartline, 1933; Victor, 1935; Waddington *et al.* 1936 and Duryee, 1936]. An entirely different method described by Linderstrøm-Lang [1937] and extended by Needham & Boell [1939], is based on the principle of the Cartesian Diver.

While each of these types possesses certain advantages, it appears that none of them in their present form can carry out on a micro-scale all the kinds of measurement which can be done on a larger scale in the Barcroft or Warburg apparatus.

The apparatus described below employs a principle which has so far not been used in a respirometer. As regards sensitivity, it is capable of measuring uptakes and outputs of about 1 μl./hr. with an accuracy at least as great as that of the Warburg apparatus; it is therefore less sensitive than some of the instruments described above. However, unlike these others, it possesses all the potentialities of the Warburg apparatus. It can be assembled at one temperature and used at a widely different one; it can be filled with any gas mixture, and two or more separate fluids contained in it can be mixed at any desired time during an experiment. The fact that it can be used under sterile conditions is an additional advantage.

Principle

The respiring material rests in a completely closed chamber, one wall of which consists of a thin sheet of mica. Two plane mirrors are attached to the latter, and when the volume of the gas in the chamber changes, the mica will bulge (either inwards or outwards) with the result that the mirrors will tilt in opposite directions. By applying a positive or negative pressure to the *outside* of the mica membrane, the latter may be brought back to its original position, a simple optical system being used to detect when the mirrors are in the same plane. Then, knowing the volume of the gas space and the change in pressure in it, the amount of gas absorbed or given out may be calculated.

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CONSTRUCTION

The component parts of the apparatus may be described under five headings:

(1) The chamber containing the tissue.

(2) The metal case containing the chamber.

(3) The optical system.

(4) Thermostat, with heaters, thermoregulator etc.

(5) The pressure-regulating mechanism and manometer, and arrangements for filling the chambers with any desired gas mixture.

(1) *The respiration chamber.* This consists of two plane slabs of glass 25 mm. square, one 3–4 mm. thick, and the other about half this thickness. The thicker of the two—which will be referred to in the rest of this paper as the "cup"—has a cavity of 40 to 80 μl. capacity in one side, in which lies the material being studied. The inside of the cavity, which may be of any shape, is lined with wax, so that two or more separate drops of fluid may be placed in the different arms of the cavity without mixing. (Fig. 1*a* shows a "cup" capable of holding

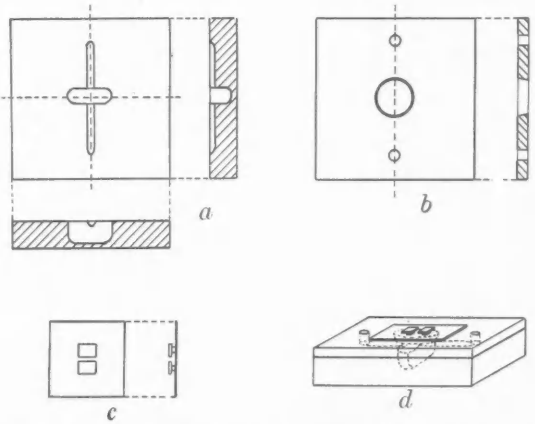

Fig. 1. Components of respiration chamber. *a*, "cup". *b*, "plate". *c*, mica membrane with mirrors attached. *d*, complete assembly.

two separate drops of fluid; Plate I A shows "cups" for two, three and four drops of fluid.) The "cup" also has two grooves cut in the plane surface, which lead to the cavity, as shown in Fig. 1*a*.

The thinner slab—referred to in this paper as the "plate"—has a central hole about 7 mm. in diameter, which is covered on one side by the mica membrane. The "plate" (Fig. 1*b*) also has two smaller holes, 18 mm. between centres, which coincide with the ends of the grooves in the "cup" when the two are in apposition. Fig. 1*d* shows a complete assembly of "cup", "plate" and mica ready to be placed in the brass case.

The respiration chamber may be filled with any desired gas mixture by leading the latter through one hole in the "plate". (The way in which this is done will be understood after reading the following section.) The gas enters the respiration chamber through one groove and leaves it by the other, and after a sufficient stream of gas has been passed, the chamber can be completely closed by rotating the "cup" relative to the "plate".

The "cup" cavities can be made by drilling a number of holes in the glass slab with a china-riveter's diamond drill [Heatley, 1938], and then enlarging them and running them together by means of a suitably shaped carborundum dental burr; the holes in the "plates" are made with the same tools. Up to the present the "plates" have been made from microscope slides carefully selected for flatness of surface; optically worked glass has been used for the "cups",[1] but good quality plate glass can also be used.

The mica for the membrane should not be thicker than 18 μ, preliminary tests having shown that even very much thinner sheets are apparently quite impervious to gases. A freshly split mica sheet of the required thickness is divided into squares of 12 mm. side, by cutting it between two sheets of paper—preferably squared paper—with scissors. The mirrors are made from no. 3 cover-glasses, which are silvered, or better, aluminized, and cut up into rectangular pieces measuring 2×3 mm. These are stuck to small pieces of cover-glass 1 mm. square, which in turn are fixed to the mica (Fig. 1*c*); the insertion of the glass stalk makes the component less fragile and more sensitive. It is a considerable help when cutting the cover-glasses with a diamond chip, to lay them on a piece of oiled silk on plate glass. Bakelite varnish no. V–5209/2 has been used for attaching the mirrors to the mica, and though they easily become detached, a more suitable adhesive has not yet been found. (Cements which cannot be autoclaved, or which are affected by water, have not been tried.)

(2) *The metal case.* Considerable latitude is permissible in the design of this component, and the drawings which are appended are of the cases which have actually been used by the authors.

Fig. 2 is self-explanatory. The main body of the case is built of the thick brass disk *A*, to the back of which a thin disk *B*, having a central square hole $25{\cdot}5 \times 25{\cdot}5$ mm., is permanently fixed by two countersunk screws. A detachable disk *C*, having a central hole about 22 mm. in diameter, has three curved slots through which the three threaded rods *D* on the main body of the apparatus will pass. Projecting from one surface of *C* are eight pins enclosing an imaginary square of 25·5 mm. side. *A* has a central hole *E*, 7 mm. in diameter, which is closed on the outside by a glass window. The brass rod *J* acts as a handle, and by means of a hook soldered to it the case can be suspended from a horizontal bar fixed across the thermostat.

The "cup", "plate" and mica having been assembled into a unit (Fig. 1*d*), are placed mica downwards on to the main body of the case, which is held horizontally as in Plate I A, *b*, so that the mirrors lie in the central hole *E*, facing the glass window. The rotating back *C* is now placed on top of the glass parts with the eight pins surrounding the "cup", and the plain and spring washers are slipped on to the rods *D*, followed by the nuts *F*, which are tightened. It will now be seen that with the help of the two partially sunk rubber washers *I*, the two outer holes in the glass plate are making gastight contact with the tubes *G* and *G'* of the case, enabling gas to be passed through the respiration chamber. Fig. 3 shows the components of the whole assembly in an expanded diagrammatic form.

The slots in the disk *C* allow it to be rotated through an angle of 60°, and the pins projecting from it will force the cup to rotate with it. On the other hand the "plate" is partially sunk into the square hole in *B* and so will be held stationary. Thus the rotation of the "cup" relative to the "plate", which is required to close the respiration chamber, is carried out with ease

[1] Blanks of optically worked glass suitable for the "cups" may be obtained from Henry Hughes and Son, Ltd., Husun Works, New North Road, Barkingside, Essex.

and accuracy. Plate I A shows a photograph of some of the components already mentioned.

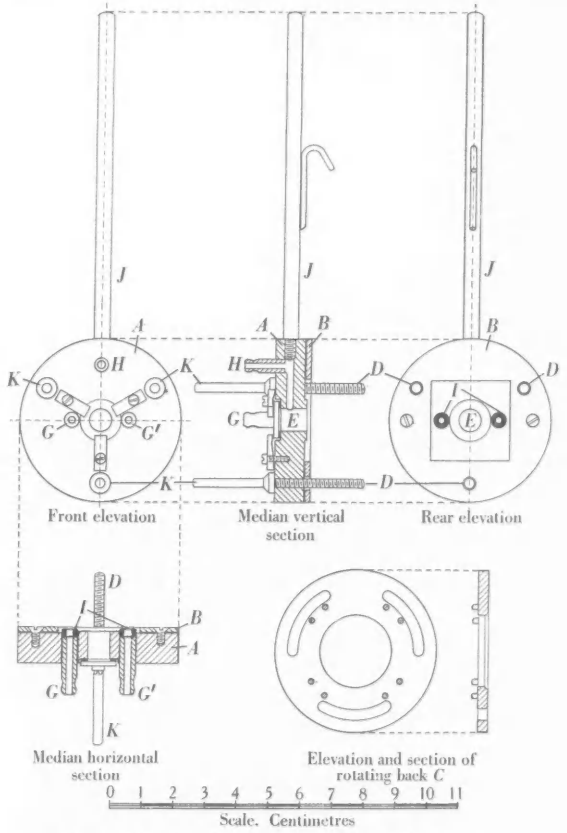

Fig. 2. Diagram showing construction of brass case.

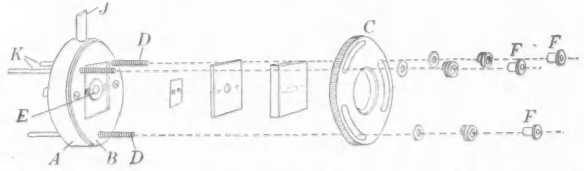

Fig. 3. Diagram showing disposition of components of brass case and respiration chamber.

In Fig. 2 will be seen a third tube, *H*, which leads directly into the space *E* between the glass window and the mica membrane; this tube is attached to the manometer and pressure-regulating apparatus, by which any bulging of the

mica can be corrected. (The bulging is detected by the optical system described in the next section.)

Some simple method for rapidly and accurately aligning the metal case with the optical system (when readings are to be taken) is required. This is done by placing the legs K of the case against a glass observation window let into the side of the thermostat, and preventing the case from rotating or sliding in a plane parallel with the window by a fixed V-shaped plate; all that is required now is some means for pressing the case against the window, and one simple mechanism for doing this is shown in plan and elevation in Fig. 4. The observation window is a small yacht decklight which is fixed to the thermostat wall by five bolts, three of which project inside and are used to secure the brass aligning-plate L. This has a V-shaped cutaway portion in which rest the legs K of the case. A stout pillar M, attached to this plate, is linked by the two freely pivoted strips N and N' to the rod O. An extension spring of stainless steel, anchored to suitable positions on the strips N and N', ensures that O will press firmly against the back of the respirometer case. O is conveniently controlled by a knob (above the level of the water in the thermostat) which is rigidly connected to N (or N') by the rod P. (In Fig. 4 the decklight, is shown in section in the plan and in dotted outline in the elevation.)

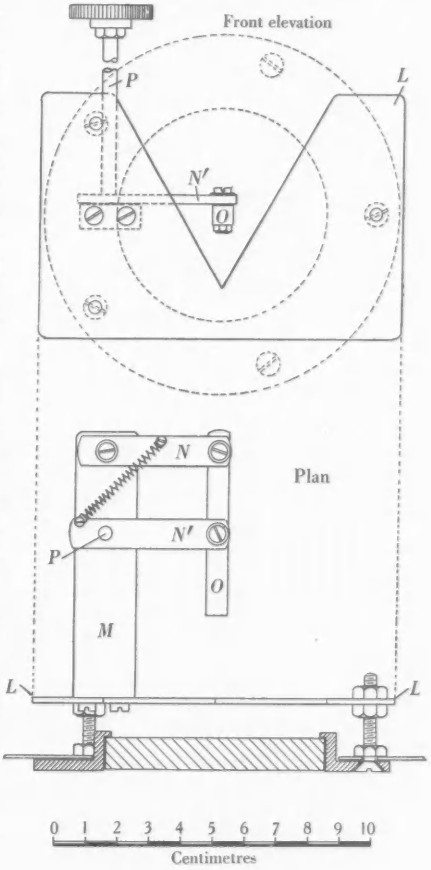

Fig. 4. Mechanism for holding brass case.

(3) *The optical system.* This is shown in its simplest form in Fig. 5*a*. An achromatic converging lens V is placed in front of an illuminated slit S so that an image of the latter would be formed, say, 70 cm. behind the lens. The mirrors W of the mica membrane are placed in the path of the convergent rays, some of which are reflected back to form an image on a ground glass screen X. When the mirrors are in parallel planes a single image of the slit will be seen, but as soon as the mica bulges one way or the other, the mirrors will tilt in opposite directions and the single image will split into two.

Even when special care is taken in the mounting of the mirrors on the mica, it will be found that only in a few cases are the planes of the mirrors approxi-

mately parallel; in the remaining cases the two images of the slit will be wide apart, and the zero position (i.e. when the two images fuse into one) can only be obtained when the mica is made to bulge. It is obvious that the membrane is most sensitive to deformation when it is flat, the sensitivity falling off rapidly as the bulging becomes greater. To avoid this loss of sensitivity a compensating arrangement of mirrors is provided by which the two images may be made to coincide, even when the pencils of rays from the two mica mirrors are widely

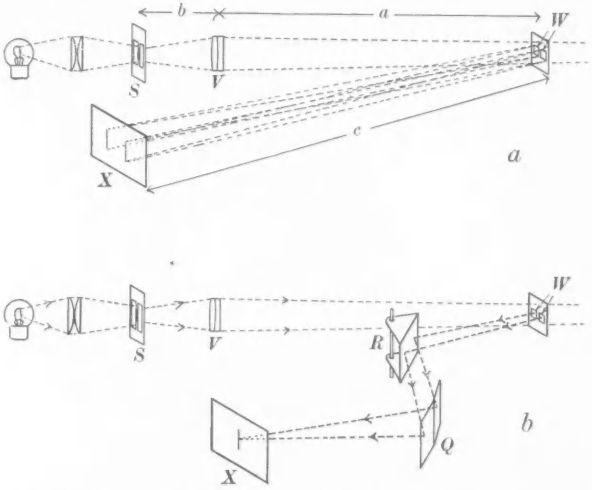

Fig. 5. Optical systems: *a*, in simplest form; *b*, with divided mirror.

divergent. Fig. 5*b* shows the principle of the method employed. Before falling on the ground glass screen, both pencils of rays are reflected from the vertically divided mirror Q, one half of which can be rotated about the dividing line. The tilt of the mirrors attached to the mica may be such that both pencils of reflecting rays, although diverging, may fall on the same half of the divided mirror Q. This contingency is met by interposing between the mica and Q another rotating mirror R, which can be so adjusted that one ray will fall on each half of Q.

When initially divergent rays are made to form a single image by the use of the divided mirror Q, there will be two different pressures which can give a zero reading, according to whether the rays are crossed before impinging on Q or not. The correct reading is easily selected, but this point may cause confusion at first. (At the correct reading a small change in pressure will cause the images to move a considerable distance relative to each other—at the other null point the same pressure change will cause only a small displacement of the images.)

The illuminated slit was found to be more efficient than a straight filament lamp.

The relation of the components of the optical system must be a matter of compromise. The distance c (Fig. 5*a*) must be great in order to obtain an efficient optical lever; but if it is made too big relative to a the area of the mirrors W will be small at that position in relation to the cross-sectional area of the converging beam, and much light will be lost. If, however, b is large, the lens will collect only a small amount of light from the slit; but if b is too small in

relation to $a+c$, the magnification of the slit will be excessive and loss of definition and brilliancy of the ground glass image will be the result.

Some light will be reflected from the window of the brass case, causing a secondary image, and another image may be formed in the same way from the thermostat window. The first unwanted image may be eliminated by having the small window in the case bedded down on a washer of uneven thickness, so that it is no longer perpendicular to the optical axis. The second may be eliminated by inclining the case at, say, 5° to the vertical, either by slightly lengthening the bottom leg K, or preferably by a suitable packing, such as a microscope slide, between it and the thermostat window. The optical system must be tilted a corresponding amount so that its optical axis is still perpendicular to the two mica mirrors W. The thermostat window will now no longer be perpendicular to the optical axis, and any reflexions from it will not interfere.

The exact arrangement of the optical system will depend on the characteristics of the individual components, but Fig. 6 shows a horizontal section through the optical box of the instrument at present in use. An image of the filament of the 36 W. 12 V. car headlamp bulb U is focussed on the vertical slit S by means of the condenser T. The achromatic lens V has an approximate focal length of 9 cm., and throws a beam of light from the slit on to the mirror R. This rotates about a vertical axis and is controlled by the screw Y, backlash being prevented by a spring. The beam of light passes through the thermostat window (shown in section in Fig. 6) and falls on to the two mirrors mounted on the mica membrane, from which two pencils of light are reflected back on to R and thence to the divided mirror Q. When the pencils of rays are initially divergent, Y may be adjusted so that one pencil of rays falls on to each half of the mirror Q; then by tilting one half of the latter by rotating the graduated drum Z (which is fixed to a threaded rod) the two separate images of the slit may be made to superimpose. Mirror X is merely for the sake of convenience, as it allows the ground glass screen to be at the front, instead of at the back of the apparatus; the screen is situated above the drum Z, and therefore is not shown in Fig. 6. In Plate I B the hood which screens it from stray light while it is being viewed can be seen. A ball bearing hinge such as that described by Baker [1938] is used for the divided mirror Q.

(4) *Thermostat, etc.* The tank at present in use is made of copper and measures 65 cm. long by 28 cm. broad by 25 cm. high. It is heated electrically by three immersion heaters of 250 W. each, and one kettle unit taking either 100 or 700 W. The thermoregulator is of the ordinary toluene-mercury type, the toluene-containing part being thin-walled glass tubing about 1 cm. in diameter. Part of the heating current is controlled by the thermoregulator, and part is fed continuously; the advantage of this arrangement—which is well known, but not used as often as it might be—is that heat is continuously supplied to the bath at a rate which would allow it to cool only very slowly; hence the intermittent heating current need only be small, and fluctuations in temperature caused by thermoregulator lag, etc., will be correspondingly reduced. The thermostat can be warmed from room temperature to 37° in about an hour by employing the 700 W. heater and one of the 250 W. ones. When the bath has warmed up, the heaters are connected as follows:

Intermittent (controlled by relay): 700 W. heater in series with one 250 W. heater.

Continuous: two 250 W. heaters in series.

In a very warm or very cold room another of the many possible heater combinations may be more suitable.

The thermostat is stirred by a three-bladed vane driven by an electric gramophone motor situated under the tank. The stirring is efficient, noiseless and trouble-free, and by having the motor under the tank (private communication from Prof. R. A. Peters) more room is left at the top, and troublesome pulleys are avoided.

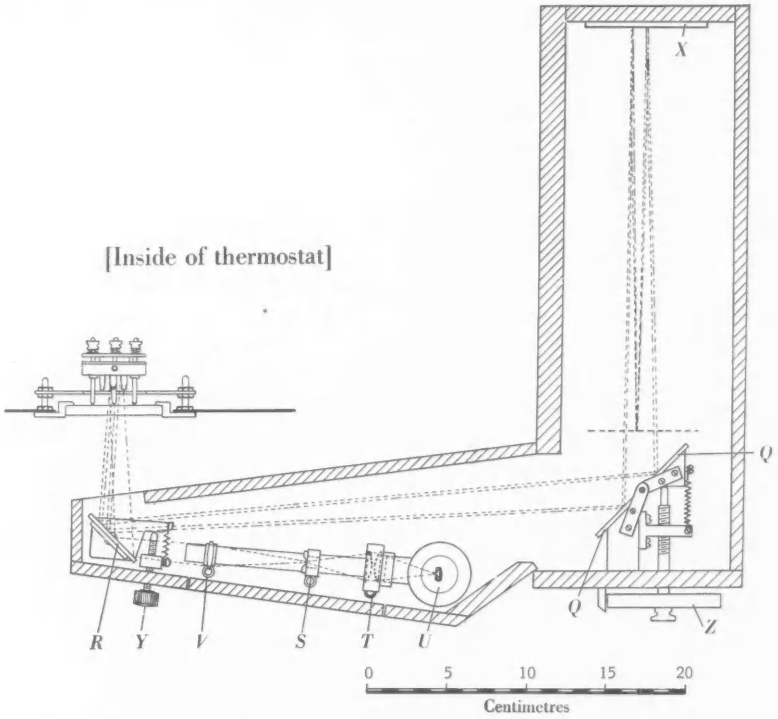

Fig. 6. Horizontal section through optical box.

A transformer giving an output of 3 amp. at 14 V. is housed behind the control panel (Plate Ib). This feeds the 36 W. car headlamp bulb used in the optical system, and also a low tension metal rectifier which delivers 2 amp. at 9 V.; a three-way switch enables either of these circuits, but never both, to be connected, whilst the third position of the switch is "off". The current from the rectifier activates the electromagnet by which two drops in the respiration chamber may be mixed (for details of this see below). An intermediate transformer tapping at 4 V. supplies current for working the thermoregulator relay, which is also situated behind the control panel.

(5) *The manometer and pressure-regulating apparatus, etc.* The principle of this is shown in diagrammatic form in Fig. 7. In the actual instrument the two reservoirs, consisting of 3 oz. bottles suspended horizontally, are attached to opposite sides of an endless chain passing round a system of pulleys and sprockets. A control wheel fixed to one of the latter enables the pressure in the system to

be varied (see Plate I B and Fig. 7). Those outlet tubes which are not connected to tube *H* of the brass cases (see Fig. 2), are closed by rubber caps. It is convenient to read the level of fluid in both arms of the manometer, thus avoiding errors through drainage; the manometer scale should be at least 60 cm. long if the instrument is ever to be used for respiratory quotient determinations (to be described in a subsequent paper).

For filling the cups with any desired gas mixture, a main tube having six side tubes is connected to the gas source; a separate bubbler, containing water and immersed in the thermostat, is interposed between this manifold and each respiration vessel, partly to moisten the gas, and partly to enable the rate of passage of gas in each individual case to be checked. It is important that the gas should be as nearly saturated as possible before entering the vessel, as otherwise evaporation of the droplets may occur, with alteration in the volume of the fluid and thus of the vessel constants. It was found that when dry O_2 was passed through the respiration chambers via the bubblers, for 10 min. at 37° at the rate of 5–10 ml./min., only about 0·5 μl. of fluid was lost from a drop of 12 μl. of distilled water. However, though this would cause a negligible error in the vessel constants, it might have a deleterious effect on delicate cells by making the medium hypertonic. The evaporation can be reduced still further by inserting a plug of damp cotton wool in the rubber tube connecting the case to the bubbler.

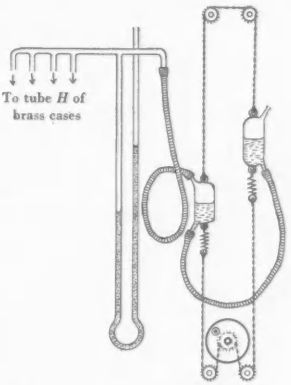

Fig. 7. Diagram showing principle of manometer and pressure regulator.

A thermobarometer is required, as in the Warburg technique, to compensate for any changes in thermostat temperature or barometric pressure. Since the volume of the vessel used as thermobarometer is immaterial, a simple bulb connected to a manometer of capillary tubing serves the purpose. Apparently no error is introduced by the difference in shape or size of this vessel compared with the respiration chambers, though should temperature regulation not be good, errors would undoubtedly arise.

Calibration and procedure

Grease. The respiration chamber is made airtight by films of grease between the component parts. Many different kinds of grease were examined and found to be unsuitable on account of their physical or chemical properties (consistency, absorption of gases etc.). For working at 37° B.D.H. winter grade rubber grease was found to be entirely satisfactory. The exact method of applying the grease is important, and too much or too little may spoil the experiment. It is most suitably applied with the ball of the finger, being patted on, rather than spread or smeared. Of the surface to which the mica is to be fixed, only a small central area need be greased, and before the mica is placed in position the edge of the central hole should be wiped free from excess grease, and this is most easily done with the ball of the clean little finger; if this is omitted, a ridge of grease may be squeezed into the cavity which will prevent the mica from moving perfectly freely.

Effect of concentration of alkali, etc. In all microrespirometers where the gas volume is small, and the surface/volume ratio of the fluids is great, isothermal distillation from one drop of fluid to another may occur; if the different drops have different initial osmotic pressures, they will tend during the course of the experiment, to attain the same osmotic pressure, with resulting changes in the concentration of the solutes in each drop. This can easily be demonstrated by actual measurement of the volumes of the drops before and after the experiment, and at 37° changes of this kind may occur in a relatively short time.

An important example of this effect is seen when strong alkali is used to absorb CO_2. This is illustrated by the results of an experiment in which the O_2 uptake of a suspension of lymphocytes was measured in the presence of (*a*) 1% and (*b*) 10% NaOH. The actual values obtained were

(*a*) In 1% NaOH: 0·25, 0·21, 0·23 μl. O_2/hr.

(*b*) In 10% NaOH: 0·14, 0·13, 0·19 μl. O_2/hr.

It will be seen that the rate of O_2 uptake is markedly lower in the latter case. It seems that lymphocytes are considerably more sensitive to osmotic changes than other tissues, but in any experiment where concentration of medium is to be avoided, strongly hypertonic solutions of alkali, etc., should not be used.

This is a point which has sometimes been overlooked in previous work with microrespirometers, and the results obtained may therefore, in some cases, be erroneous.

Method of mixing drops. One or more of the droplets inside the respiratory chamber may be mixed by dragging a small iron ball from one to the other by means of an electromagnet. Mr Leemans has invented a method of applying a thin coating of glass to small balls of a special magnetic alloy ("platinersatz-draht")[1] but an alternative method of protecting the mixing ball from corrosion is as follows. Steel bearing balls, $\frac{1}{16}$ in. in diameter, are given several coats of Bakelite varnish no. V–5209/2, each coat being polymerized by stoving before applying the next. The balls are then heated to 100° in paraffin wax (M.P. 50°) for some minutes, the surplus wax being removed by rolling the balls on hot filter paper; by this means any flaws in the Bakelite coating are blocked with wax. They are then rolled in the palm of a warm, but clean and dry hand with some well washed kaolin; this is necessary because the paraffin-coated balls are otherwise not readily wetted, and hence are inefficient mixers. As Bakelite is attacked by certain reagents (e.g. $KMnO_4$), balls prepared by this second method cannot be used in the presence of such substances. Other ways of protecting steel balls from corrosion, such as gold or nickel plating, have been tested and found unreliable.

Cleaning and preparation of the apparatus. At the conclusion of an experiment the mica membrane is dragged off the "plate" and placed on a piece of clean filter paper. The adherent grease is removed from the upper surface by means of a blunt scalpel, and from the under side by moving the mica over the surface of the filter paper. The "plate" and "cup" are then cleaned in hot chromic acid, or better, in sulphuric-nitric acid mixture. They are rinsed under the tap, then in distilled water, and dried in the oven. The "cup" cavities are then lined with paraffin wax (M.P. 70°) by carefully heating them over a small flame and touching minute shavings of wax on to the hot surface; before the "cup" has cooled completely it is rubbed face downwards on a piece of clean silk in order to remove any wax which may have spread from the cavity to the plane surface.

[1] Mr Leemans, who is glassblower at the Clarendon Physics Laboratory, University of Oxford, is prepared to supply these balls.

In preparing for an experiment the "plates" are greased in the manner described above, and the mica squares carrying the mirrors are placed in position on them. The greased "plates" (with mica membrane attached) are best kept on a special stand protected from dust until they are required. As soon as each cup is charged with the material being studied, it is covered with a plate. The flat raised rim round the central hole *E* (Fig. 2) in a brass case is carefully wiped and greased, the inner edge being wiped free from excess grease by the ball of the little finger. The "cup"-"plate"-mica assembly is placed in position, the rotating back is added, followed by the plain and spring washers and the knurled nuts which are screwed up evenly all round. At this stage the numbers of the "cup" and "plate" are noted; to reduce the possibility of mistakes, the "cups" are numbered in Roman, and the "plates" in Arabic, characters. Each knurled nut is unscrewed one half turn to allow the back of the case to be rotated more easily, and the whole is placed in the thermostat and connected to the manifold leading to the manometer, and also to one of the bubblers. At some time during the passage of the gas, the case is clamped against the thermostat window and the zero point is examined. If the two images of the slit on the ground glass screen can be made to coincide by a pressure of not more than, say, ± 30 mm. of water, nothing further is done; but if a bigger pressure is required (i.e. if the mirrors on the mica are far from parallel), the pressure is kept at zero and the two images are made to coincide by adjusting the mirror *Q* by means of the calibrated drum *Z*. The drum reading is noted, and whenever a reading is taken of that particular respiration chamber, the drum is always set to the same reading.

When all the zero readings have been taken, the gas stream is stopped and the vessels are closed by rotating the back of each case backwards and forwards five or six times; after 2 or 3 min. temperature equilibration is attained and readings may be commenced. Each case has three rubber tubes leading from it, and when several cases are being used it is convenient to keep these tubes out of the way by suspending them in hooks attached to elastic cords which dangle from a permanent framework. The latter and the elastic and hooks can be seen in Plate I B.

Determination of constants. The constants of the respiration chambers are determined as for the Warburg vessels [*vide* Dixon, 1934]. The constant, *K*, is therefore given by the formula

$$K = \frac{v_G \frac{273}{t} + v_F \alpha}{P_0},$$

where v_G = volume of gas space,

v_F = volume of fluid,

α = solubility coefficient of the gas in the liquid concerned,

t = the absolute temperature,

P_0 = the normal pressure (760 mm. Hg) in mm. of manometric fluid. If *D* is the density of the latter, then $P_0 = \frac{760 \times 13{\cdot}6}{D}$.

Then, where h is the manometric reading observed, the amount of gas, x, evolved or absorbed $= hK$.

The volume of fluid, v_F, can be measured by a pipette, or by determining the increase in weight of the tared "cup" after adding the fluid.

The total volumes of the "cups" and "plates" are determined initially by weighing the amount of mercury required to fill completely the central space. In the case of a "cup" this is done by filling with mercury, laying a plane slab of glass on top, and squeezing out the excess of mercury. Adhering globules

of the latter are brushed off, and the mercury is transferred to a tared watch-glass, which is reweighed. The volume of the central cavity of a "plate" is found in the same way, except that the "plate" is squeezed between two slabs of plane glass. The volume of the gas space, v_G, is obtained by subtracting the volume of fluid, v_F, plus the volume of the mixing ball (if used) from the total volume.

A number of factors suspected of altering the effective volume of the whole assembly were investigated, since any alteration in the volume of the vessel will introduce corresponding errors in the constant. The first of these factors was the thickness of the film of grease between the components. The error from this cause is negligible, since bright interference colours can always be seen as soon as air enters between the faces of the "cup" and "plate" when these are wrung apart after an experiment. The second and third possible sources of error are (*a*) the volume of the wax used to line the cavity of the "cup", and (*b*) the volume of grease which will be squeezed into the respiration chamber when it is screwed up tightly in its metal case. No allowance is made for either of these factors, but quantitative investigations showed that from these two causes the results may be anything up to 3% too high.

Accuracy of the constants. This was investigated by measuring the volume of O_2 liberated from a known amount of H_2O_2 by means of catalase, control experiments being carried out in the Warburg apparatus. The results are shown in Table I, the calculated values being obtained from titrations with $KMnO_4$. The

Table I. *Test experiments with H_2O_2*

In each case approximately 1 μl. of gas was evolved in the microrespirometer

Exp.	Vol. O_2/ml. H_2O_2 solution: Microrespirometer	Warburg	Calculated
1	204 208	—	202
2	198 200	—	216
3	78 77 77 77 80	78 81 81	—
4	81 74 75 79	77 77 78 79 80	84·5
5	79 85 79 82	77 80 81	82·5

occasional discrepancies are almost certainly due to the fact that the weak H_2O_2 solution decomposes slowly during the equilibration period. Another series of experiments was carried out in which the volume of CO_2, liberated from bicarbonate solution in an atmosphere containing 5% CO_2 by a known amount of acid, was measured. The results are shown in Table II. The scatter in these experiments is due to the fact that it is difficult to get the "cup", and more particularly the mixing ball, completely free from traces of acid or base. We are convinced that neither of the two sets of figures given is a true indication of the accuracy of the instrument when used for measuring O_2 uptakes.

Table II. *Measurement of volume of CO_2 displaced from bicarbonate by a known amount of acid*

Calculated value, 0·90 μl.

Exp.	Vol. CO_2 evolved μl.	% error
1	0·83	−8
2	0·85	−5·5
3	0·90	0
4	0·90	0
5	0·82	−9
6	0·87	−3·5
7	0·84	−6·5
8	0·87	−3·5
9	0·85	−5·5
10	0·90	0
11	0·96	+6·5
12	0·87	−3·5
13	0·89	−1
14	0·88	−2
15	0·89	−1
16	0·92	+2

The role of diffusion. In most of the previously mentioned respirometers dealing with uptakes of the order of 1 μl. O_2/hr., shaking is omitted on the assumption that diffusion of the gas in the droplet will be adequate. This point was investigated by comparing the rate of O_2 uptake in the microrespirometer with that in the Warburg apparatus as control. An inorganic system such as a spontaneously oxidizable titanous salt is unsuitable for this purpose, because the rate of O_2 uptake will be proportional to the partial pressure of O_2; as this will decrease towards the centre of the droplet, the rate of uptake of the inside of the drop will be less than that of the outside layer. What is required is a catalytic system in which the catalyst has a high affinity for oxygen, and experiments showed that kidney amino-acid oxidase with alanine as substrate [Krebs, 1935] was satisfactory in this respect.

A large number of experiments was carried out with this system in an atmosphere of O_2, with droplets between 4·0 and 16·0 μl. in volume, with total uptakes ranging from 0·5 to 2·7 μl. O_2/hr. Of the 59 microrespirometer experiments which were done, the standard deviation of the percentage error was 4·8, a similar scatter being obtained with the Warburg values. The agreement, in any particular series, between the two instruments was in most cases within $\pm 10\%$, and though occasionally even worse results were obtained, it is quite clear that in an atmosphere of O_2 there is no effect attributable to diffusion.

Comparative experiments in air, however, showed a definite effect of diffusion, for considerably lower values for the rate of O_2 uptake were obtained with the microrespirometer than with the Warburg apparatus, with drops down to 4·0 μl. in volume having an uptake of 0·4 μl./hr. Even when the drop was suspended in a platinum loop inside the vessel, so that maximum surface was exposed to the gas mixture, the values were low by as much as 20%.

In view of the results obtained in an atmosphere of air, it was thought desirable to test rates of uptake in O_2 under more stringent conditions. Tyramine oxidase has been shown by Kohn [1937] to be highly dependent on the O_2 pressure, and some comparative experiments with this system were carried out in the microrespirometer, with the Warburg apparatus as control, with the results shown in Table III. The activity of the extract fell off rapidly, so that accurate comparison of the results in the two instruments was not possible. The

Table III. *Experiments with tyramine oxidase*

In all experiments the volume of extract taken in the microrespirometer was 10·3 μl., but this quantity was sometimes divided into two or three separate droplets in the respiration chamber.

Exp.	O_2 uptake/hr./μl. extract		Number of drops of fluid in microrespirometer
	Warburg	Microrespirometer	
1	285	306	2
	285	291	1
	303	264	2
		260	1
2	310	291	3
	320	276	3
	280	310	1

table shows clearly, however, that even when oxidative systems are used which are dependent on O_2 pressure, the rates of O_2 uptake of these systems can safely be measured in this instrument. Since correct values are obtained in O_2 with relatively large drops and rapid rates of uptake, it might have been expected that normal values would also be obtained in air, provided that the drop of fluid were sufficiently small. This is not the case and therefore factors other than simple diffusion must be operating to account for this phenomenon. However, a discusion of these other factors is beyond the scope of the present communication.

Comparative experiments, with the Warburg apparatus as control, have also been done on the rate of liberation of CO_2 from urea by the action of urease; entirely satisfactory agreement between the two instruments was obtained.

The apparatus described in this communication is suitable for the measurement of rates of O_2 uptake and other metabolic activities of small pieces of surviving tissues. As, however, the case of small pieces of tissue involves a number of special considerations and the application of special methods, this question is treated in detail in the following paper by Berenblum *et al.* [1939].

Details for the measurement of respiratory quotients and aerobic glycolysis in this instrument will be given in a subsequent paper.

Summary

1. A new type of microrespirometer is described which will measure O_2 uptakes of the order of 1 μl./hr., with a standard error similar to that normally obtained with the Warburg apparatus.

2. The respiration chamber can be filled with any desired gas mixture, and two or more separate fluids in it may be mixed at any time during the experiment. The apparatus may be charged at one temperature and used at a widely different one, and it can be sterilized.

3. The estimation of substances which can be determined manometrically in the Warburg apparatus can also be carried out in the microrespirometer, the amounts which can be estimated being approximately 200 times smaller.

4. No special skill is required in its manipulation, and the setting up of an experiment takes only slightly longer than in the case of the Barcroft or Warburg apparatus. During the course of an actual experiment six readings can be taken in 5 min., with a little practice.

We wish to thank Dr H. G. Kuhn and Dr P. E. Lindahl for helpful suggestions. Our thanks are due to the Medical Research Council for a personal grant and a grant for apparatus to one of us (N. G. H.), and to the British Empire

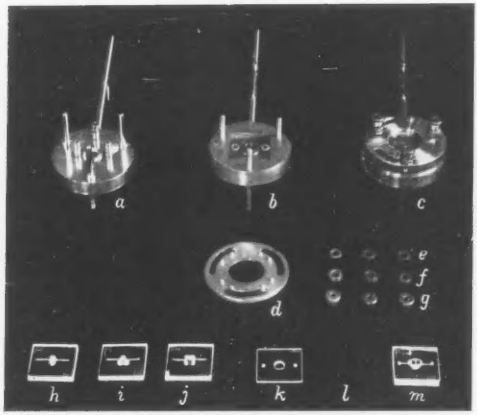

Plate I A

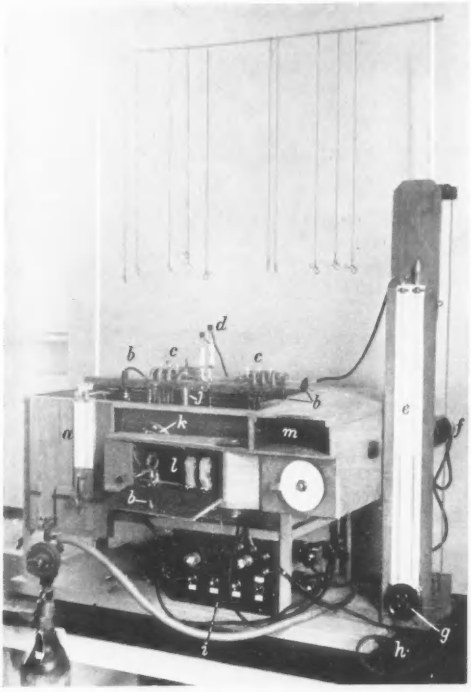

Plate I B

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Cancer Campaign for personal grants to two of us (I. B. and E. C.). Preliminary experiments were carried out by one of us (N. G. H.) at the Biochemical Laboratory, Cambridge, England, during his tenure of the Benn W. Levy studentship.

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EXPLANATION OF PLATE I

PLATE Ia

Components of the respiration chamber and brass case. *a*, brass case, face upwards. *b*, brass case, face downwards. *c*, complete assembly ready for immersion in thermostat. *d*, rotating back of case, showing pins. *e*, plain washers. *f*, spring washers. *g*, knurled nuts. *h*, *i* and *j*, "cups" with cavities for two, three and four separate drops of fluid respectively. *k*, "plate". *l*, mica, with mirrors attached. *m*, complete respiration chamber ready for insertion in brass case.

PLATE Ib

General view of complete apparatus. *a*, thermobarometer. *b*, immersion heaters. *c*, bubblers and manifold leading to manometer, etc. *d*, thermoregulator. *e*, manometer. *f*, one of the reservoirs of the pressure-regulating mechanism. *g*, crank actuating pressure-regulating mechanism. *h*, electromagnet—for mixing drops. *i*, control panel. *j*, top of thermostat stirrer. *k*, window in side of thermostat. *l*, part of optical system (lens, slit and condenser) seen through opened panel of optical box. *m*, hood protecting ground glass screen from stray light.

Note. The bar from which the brass cases are suspended is not shown in this photograph.

IX. THE STUDY OF METABOLIC ACTIVITIES OF SMALL AMOUNTS OF SURVIVING TISSUES

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(*Received 29 October 1938*)

The microrespirometer described in the previous communication is applicable to the measurement of metabolic activities of small amounts of surviving tissues (e.g. for histochemical studies) and of cell suspensions. In the present communication certain features of technique in micro-manometric studies of the metabolism of minute fragments of surviving tissue are described and consideration is given to special difficulties which arise in such studies.

Preparation of tissues for the experiment

The area of tissue slice which can conveniently be used in the microrespirometer is approximately 0·5–8·0 sq. mm. This size depends upon the metabolic activity of the particular tissue used and must be such as to give a gas exchange of not less than 0·3 μl. and not more than 3·0 μl. per hr.

The small slices are obtained by cutting a number of ordinary tissue slices from the fresh organ with a sharp razor in the usual manner. One of these, chosen for its thinness and uniformity, is then placed on a microscope slide and cut into small squares with a sharp razor. These tissue pieces are then washed from the slide into a small dish with the required medium and transferred from the dish to the respiration chamber by means of a very fine platinum spatula. Careful handling of these small tissue slices is essential if damage and consequent decrease in the rate of metabolism is to be avoided. The excess medium is removed with a fine capillary and a known amount of fresh medium is added by means of a micro-pipette.

In the case of suspensions of cells such as bacteria, yeast, blood cells etc., a known amount of the suspension is measured directly into the respiration chamber by means of a micro-pipette.

Cell damage caused by cutting

In tissue slices of very small size the ratio of cut surface to total volume is obviously higher than in large slices such as are ordinarily used in the Warburg apparatus. It might therefore be argued that the higher proportion of dead cells (due to the greater amount of cut surface) would result in lower metabolic values being obtained with small than with large slices.

This problem was investigated by comparing in the Warburg apparatus the rates of O_2 uptake of ordinary tissue slices and of similar slices cut up into small squares (as required for the microrespirometer), using liver, kidney and Jensen rat sarcoma for the purpose. The results summarized in Table I show that there is no significant difference between the two series. (The occasional low results with cut up slices of kidney are attributable to a peculiar clumping effect which sometimes occurs when several small pieces of kidney are shaken in the Warburg apparatus).

[1] Beit Memorial Research Fellow.

Table I. *Comparison between the Q_{O_2} values for whole and cut up slices of different organs (in Warburg apparatus)*

	Q_{O_2}*	
	Whole slices	Cut up slices
Rat liver	8·2	8·0
	9·5	9·3
	8·0	9·0
Rat kidney	17·5	16·0
	16·5	14·0
	18·0	16·3
Jensen rat sarcoma	10·0	9·5

* The figures represent average values in separate experiments, each figure being the mean of two or more determinations.

The unexpectedly good agreement between the values for whole and cut up slices is due to the fact that a certain proportion of the damaged cells on the surface is removed by the gentle agitation during the preliminary washing and that, though the amount of damaged cells is proportionately greater in the small pieces, the amount shaken off is also proportionately greater. Nitrogen determinations on samples of liquid used for the preliminary washing show that in the case of small slices at least 10 % of the initial material is lost in the washings.

Diffusion

The rate of respiration of most tissue is known to be within wide limits independent of the oxygen tension. It has been shown in the previous communication [1939] that though no provision is made for a shaking arrangement such as that used in the Warburg apparatus, the values for O_2 uptake of homogeneous extracts in an atmosphere of O_2 are the same in the microrespirometer as in the Warburg apparatus, even with oxidative systems which are strongly dependent on O_2 pressure. In an atmosphere of air, however, low values are obtained. Therefore, as far as diffusion of O_2 through the liquid is concerned, it is evident that provided the tests are carried out in O_2 and not in air, the microrespirometer without any stirring can be used safely for the measurement of oxygen uptake of tissue slices. The limiting thickness of the tissue, however, requires separate consideration.

In the respiration chamber, that part of the tissue which touches the bottom does not come into direct contact with the O_2-containing medium, and under the most unfavourable conditions (i.e. when the whole of one surface is in close contact with the bottom of the cup) almost half of the total surface of the slice will be deprived of direct O_2 supply. It may be assumed, therefore, that the limiting thickness of the tissue which may safely be used in the microrespirometer is, even under the most unfavourable conditions, not less than half of that used in the Warburg apparatus. According to Warburg [1923] in an atmosphere of oxygen the maximal permissible thickness for liver slices is approximately 0·5 mm. and that for kidney slices 0·3 mm. Since it is possible without any special efforts to cut liver slices of 0·25 mm. and kidney slices of 0·15 mm. thickness, no special difficulties need be expected on account of diffusion through the tissue.[1]

[1] The diffusion constant of O_2 through different tissues is not necessarily identical with or even similar to the value for connective tissue of the gut [Krogh, 1919], which has hitherto been generally used in applications of Warburg's formula for the maximal thickness of tissues for metabolic work. For this reason the limiting thickness of any tissue should always be determined by empirical tests.

A comparison between the rates of O_2 uptake of tissue slices measured simultaneously in the Warburg apparatus and in the microrespirometer is given in Table II. The slices were cut without any special precautions, the thinnest and most uniform pieces being chosen for the experiments. (In several cases the thickness of the pieces was estimated from measurement of their areas under the microscope and their weights. No significant differences in the Q_{O_2} values were observed in the microrespirometer experiments with slices of liver varying in thickness from 0·1 to 0·3 mm.)

Table II. *Comparison between the Q_{O_2} values for rat liver and kidney obtained in the Warburg apparatus and microrespirometer*

Tissue	Q_{O_2} Warburg apparatus	Q_{O_2} Micro-respirometer	Weight of tissue used in the microrespirometer μg.
Rat liver	8·3	6·5	173
	8·5	7·1	157
	8·3	5·5	187
	9·2	6·5	208
	8·3	7·4	210
	Av. 8·5	Av. 6·6	
Rat liver	5·2	5·0	187
	5·2	5·0	175
	4·9	4·9	207
	5·3	—	—
	5·4	—	—
	Av. 5·2	Av. 5·0	
Rat liver	13·6	9·5	95
	12·6	9·5	101
	10·3	8·2	93
	10·8	9·4	75
	10·5	—	—
	Av. 11·6	Av. 9·1	
Rat kidney	19·7	18·8	33
	15·5	18·1	45
	—	19·8	24
	—	17·3	37
	Av. 17·6	Av. 18·5	
Rat kidney	17·3	17·7	79
	15·5	20·8	78
	—	15·4	89
	—	18·8	120
	Av. 16·4	Av. 18·2	

Table III. *Effect of oxidizable substrates on Q_{O_2} values of liver and kidney in the Warburg apparatus and in the microrespirometer*

Tissue	A Q_{O_2} in absence of substrate: Warburg apparatus	A: Micro-respirometer	B Q_{O_2} in presence of substrate: Warburg apparatus	B: Micro-respirometer	Ratio B/A: Warburg apparatus	Ratio B/A: Micro-respirometer
Rat kidney	18·6	16·6	35	27	1·88	1·63
Rat kidney	18·0	18·0	31	29	1·72	1·61
Rat liver	8·0	7·2	13	12·5	1·62	1·73

The substrate used was alanine in the case of kidney and succinate in the case of liver.

Table IV. *Rate of O_2 uptake of yeast suspension expressed as μl. O_2 per ml. suspension per hr.*

Comparison of values obtained in Warburg apparatus and in microrespirometer

Experiment	Warburg apparatus	Microrespirometer
1	170	153
2	200	178
3	213	253
4	136	147

The yeast suspensions were made up as follows: 15 ml. of 1–3 % yeast suspension plus 20 ml. of 5 % glucose plus 15 ml. of *M*/15 phosphate buffer solution.
Each figure represents the mean of 3–5 determinations carried out simultaneously.

The results show close agreement between the values obtained in the two instruments for the oxygen uptake of kidney slices, but with liver slices in most cases slightly lower values were obtained in the microrespirometer. This is attributable to the fact that liver tissue, being particularly friable, continues to shed dead and damaged cells during the course of the experiment, due to the shaking in the Warburg apparatus, whereas this occurs to a smaller extent with tissues such as kidney. Since these detached cells are not weighed after the end of the experiment, correspondingly high values are obtained with liver slices in the Warburg apparatus as compared with the microrespirometer. The amount of material actually detached was determined by nitrogen estimations of samples from the supernatant fluid in the Warburg vessels taken at different time intervals. It was found to account for approximately 15 % of the total weight of tissue. If 15 % is added to the Q_{O_2} values for liver obtained in the microrespirometer (Table II), the values approach those obtained in the Warburg apparatus.

For additional evidence in support of the contention that diffusion through the tissue is adequate, experiments were undertaken on measurements of rates of O_2 uptake of liver and kidney slices with and without oxidizable substrates (succinate for liver and alanine for kidney), the experiments being conducted simultaneously in the Warburg apparatus and in the microrespirometer. The percentage increase in the rates of O_2 uptake of the slices in the presence of substrate showed close agreement in the two instruments (Table III).

Experiments were finally undertaken with yeast suspensions, in order to determine whether, even under these stringent conditions (i.e. a marked tendency for the cells to settle and a very high Q_{O_2}), diffusion would still be adequate in the microrespirometer without a shaking system. Once again determinations were made simultaneously in the Warburg apparatus and the microrespirometer, and the results, shown in Table IV, show close agreement in the two instruments.

Determination of amounts of metabolizing material

(1) *Dry weight.* The metabolic values for tissue slices obtained by means of the Warburg apparatus are usually expressed in terms of unit dry weight. The same standard can be used for the small tissue slices required for the microrespirometer, provided the tissue is sufficiently homogeneous. At the end of the experiment the small tissue slice is carefully removed from the respiration chamber by means of the fine platinum spatula, placed in distilled water for a few seconds (to remove the salts of the medium) and transferred to a tared cover slip. This is dried in an oven at 110° for 1 hr. and reweighed. The ordinary Kuhlmann type microchemical balance can be used for determinations of dry weights of 80 μg. or over, but with smaller amounts the weighing error begins

to affect seriously the accuracy of the results. In such cases the Nernst-Donau balance [Donau, 1933] is indicated.

(2) *Nitrogen determination.* As an alternative to dry weight, the N content of the tissue can be used as a standard for expressing metabolic values. Like the dry weight standard, it is only applicable to fairly homogeneous tissues. Since methods of high sensitivity for N determination are now available, the nitrogen standard is applicable to very small amounts of tissue.

(3) *Cell counts.* This has an important theoretical advantage over the previous methods in that it represents a true measure of the respiring elements of the tissue only, so that the variable amounts of non-respiring elements (keratin, collagen, products of secretion etc.) in non-homogeneous tissues will not affect the final results. In practice the cell count standard is applicable to cell suspensions, for which it is probably the ideal method. For tissue slices the method is tedious and troublesome and complicated by the difficulty of making adequate corrections for the cut cells which are counted twice in serial sections. Such a method has, however, been successfully worked out for certain cases by Linderstrom-Lang *et al.* [1935].

(4) *Nucleic acid standard.* When using tissues such as skin, thyroid, brain, necrotic tumour, tissue culture etc., in which large and variable amounts of metabolically inactive material are present, the dry weight and nitrogen standards are unsuitable, since they do not differentiate between the cellular respiring elements and the inactive non-respiring material. The metabolic values, based on such standards, are not only abnormally low but also grossly irregular, since the proportion of living to non-living material in such tissues is liable to vary under different conditions of health and disease and from one animal to another.

The standard required for expressing metabolic activities of such tissues is one which differentiates cellular from non-cellular elements. The nucleic acid content of the tissues appears to answer this purpose.

The method which has been used by the authors consists of the estimation of nucleic acid phosphorus.

Method. After removal from the respiration chamber, the piece of tissue is placed in a small tube of about 5 ml. capacity, containing about 2 ml. of an alcohol-chloroform mixture (3 : 1), and heated for 2 hr. under a small reflex condenser similar to that described by Wasitzky [1932]; this extraction is repeated with fresh alcohol-chloroform mixture for a further 2 hr. The purpose of this is to remove lipoid phosphorus. The tissue is then extracted with $N/10$ HCl in the cold for 3 hr., to remove the inorganic and organic acid-soluble phosphorus. For efficient extraction, continuous agitation is essential, and this is conveniently carried out by mounting the stoppered tubes radially on a vertical disk which is slowly rotated by a motor. The tissue is finally ashed with perchloric acid and the phosphorus estimated by the micro-method of Berenblum & Chain [1938].

The method gives reasonably accurate results with amounts of nucleic acid phosphorus down to 0·2 μg., representing approximately 1·8 μg. of nucleic acid. Table V shows some values for the nucleic acid phosphorus contents of liver, kidney and brain obtained by this method. Fujiwara *et al.* [1937] found the nucleic acid phosphorus content of rat's liver to be 0·434 %, while Javillier & Allaire [1926] give the following figures for horse's tissue: liver = 0·204 %, kidney = 0·169 % and brain = 0·075 %.

Estimations of nucleic acid phosphorus were also carried out on special tissues with the object of examining the suitability of nucleic acid as a measure of cellular material.

Table V. *Nucleic acid phosphorus contents of liver, kidney and brain of the rat*

Tissue	Nucleic acid phosphorus content, expressed as % of dry wt.
Liver	0·27, 0·37, 0·38, 0·36, 0·32 Av. 0·34
Kidney	0·30, 0·29, 0·27, 0·31, 0·29 Av. 0·29
Brain	0·09, 0·14, 0·10, 0·12, 0·11, 0·11 Av. 0·11

The estimations were carried out on small pieces of tissue varying from 216 to 885 μg. in dry wt.

Table VI. *Nucleic acid phosphorus contents of tissues undergoing autolysis*

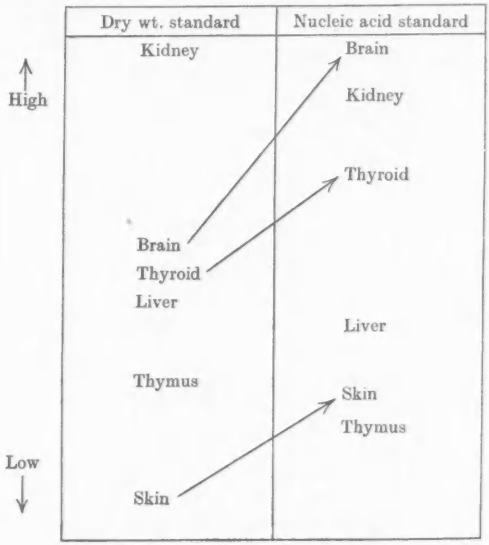
Tissue	Nucleic acid phosphorus content (as % of dry wt.)
Jensen rat sarcoma (non-necrotic portions)	0·70, 0·63, 0·84, 0·52, 0·70, 0·68 Av. 0·68
Jensen rat sarcoma (necrotic portions)	0·15, 0·18, 0·19, 0·24, 0·20 Av. 0·19
Liver (normal)	0·31, 0·35, 0·32 Av. 0·33
Autolysing liver* after 24 hr.	0·19, 0·15, 0·15 Av. 0·16
Autolysing liver* after 2 days	0·19, 0·16, 0·19 Av. 0·18
Autolysing liver* after 5 days	0·20, 0·17, 0·21 Av. 0·19
Tuberculous caseation from spleen of monkey	0·34, 0·37, 0·33, 0·34, 0·37, 0·37 Av. 0·35

* Thin slices of rat's liver floating in saline (with addition of a little toluene) kept at 37°.

Pure connective tissue, as exemplified by tendon of the rat's tail, was found to contain no significant amount of nucleic acid phosphorus. In order to investigate the rate of disappearance of nucleic acid following the death of a cell, three different examples of necrotic tissue were chosen for investigation, namely autolysing liver, the necrotic part of a tumour and tuberculous caseation. The results of these tests (Table VI) can be briefly summarized as follows.

The necrotic part of the tumour contained about one quarter of the nucleic acid of non-necrotic tumour; in autolysing liver the nucleic acid content fell off rapidly to about half of the original content and then remained fairly steady; in caseating material the nucleic acid content was found to be high.

Table VII. *Diagram showing order of the relative metabolic values* (Q_{O_2}) *of different tissues based on dry wt. and nucleic acid standards*



The results may be explained by the fact that the enzymes responsible for the disappearance of nucleic acid are themselves ultimately destroyed during the process of autolysis. In caseation, where coagulation of the whole cell content sets in quickly, the nucleic acid splitting enzymes are inactivated very early, so that the bulk of the nucleic acid remains intact.

The metabolic values, quoted in the literature [cf. Krebs, 1933], for slices of tissue, have hitherto been calculated in terms of dry weight. Since on this standard no allowance is made for inactive elements in the tissue, the values obtained cannot be expected to bear any relation to the true metabolic activity of the cells contained therein. This is borne out by the comparison between the relative metabolic values (Q_{O_2}) for different tissues based on dry weight and on nucleic acid standards (see Table VII).

From general considerations of the physiological functions of these organs and from their respective blood supplies in the intact body, the order of their metabolic activities would seem to correspond much more closely to that given by the nucleic acid standard than that based on dry weight.

Summary

1. Details of the cutting and preparation of small tissue slices, suitable for use in the microrespirometer, are described.

2. Diffusion of O_2 through the tissue was found to be adequate, provided the slices were cut reasonably thin.

3. In the Warburg apparatus tissue slices which had been cut into small pieces gave identical Q_{O_2} values with whole slices.

4. Comparative experiments on the O_2 uptake of liver and kidney slices, carried out simultaneously in the Warburg apparatus and microrespirometer, showed reasonably good agreement between the two, both in the presence and absence of oxidizable substrates. Thus, under suitable conditions, the effects of diffusion and of damage of the tissue due to cutting, are negligible.

5. Various methods for determining the amounts of metabolizing material are discussed. For tissues containing large amounts of inactive material (e.g. skin, thyroid, necrotic tumour, tissue culture etc.), it is suggested that the metabolic values be expressed in terms of nucleic acid phosphorus content. A convenient method for the estimation of nucleic acid phosphorus in small amounts of tissue is described.

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X. THE IMMUNOLOGICAL PROPERTIES OF PROTEINS TREATED WITH $\beta\beta'$-DICHLORODIETHYLSULPHIDE (MUSTARD GAS) AND $\beta\beta'$-DICHLORODIETHYLSULPHONE[1]

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SEVERAL explanations have been put forward to account for the vesicant action of mustard gas and related compounds. Cashmore & McCombie [1923] refer to the suggestion that the effect might be due to a reaction between the mustard gas and "amino-acids present in the skin". In support of this theory might be mentioned the experiments of Clarke [1912], who showed that mustard gas combines with primary amines, those of Cashmore & McCombie [1923], who found that mustard gas and $\beta\beta'$-dichlorodiethylsulphone give condensation products with glycine ester, and the observation that $\beta\beta'$-dichlorodiethyl-sulphide, -sulphoxide and -sulphone react with various primary, secondary and tertiary amines [Lawson & Reid, 1925]. The last-named authors conclude that their results are consistent with the theory that the vesicant action of mustard gas is due to its reaction with constituents of the living cell.

The introduction of new groups into the protein molecule, by attachment to the free amino-groups, leads usually to a very marked change in the immunological properties of the protein. Landsteiner & Jablons [1914] and Landsteiner & Lampl [1917] found that treatment with formaldehyde renders rabbit serum proteins fully antigenic for rabbits. Although the original species specificity is not completely destroyed, the action of formaldehyde is sufficient to produce a protein derivative which is "foreign" to the body. Horsfall [1934] obtained essentially similar results with formaldehyde-treated proteins.

Amongst other reagents which react with the free amino-groups of proteins is phenyl *iso*cyanate, and this substance destroys, almost completely, the original species specificity of serum proteins; the phenylcarbamido-proteins produced have an immunological specificity which is characteristic for the phenylcarbamido-grouping [Hopkins & Wormall, 1933, 1, 2; 1934]. Similar results follow the action of benzyl chloroformate on antigenic proteins [Gaunt *et al.* 1935; Gaunt & Wormall, 1938], for in this case also the acquirement of a new immunological specificity is related to the introduction of a new group attached to the free amino-groups of the original protein.

[1] The investigations described here were mainly carried out when the authors were members of the Departments of Experimental Pathology and of Physiology in the University of Leeds. A brief account of the earlier results was communicated in 1936 to the 2nd Internat. Congress for Microbiology (cf. *Report*, 1937, p. 427).

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In view of these immunological investigations and the above-mentioned work on the action of mustard gas and related compounds on amino-acids, it was considered desirable to study the immunological properties of mustard gas-treated proteins. The investigations described were undertaken (*a*) to determine whether serological methods would enable us to detect a reaction between mustard gas and proteins, and (*b*) to study the general immunological properties of these mustard gas- (and sulphone)-treated proteins.

Experimental

For the preparation of mustard gas- (or sulphone)-treated proteins, the reagent was allowed to act on the protein solution in the presence of phosphate buffer at *p*H 8·0. Since mustard gas is gradually hydrolysed by water with liberation of free HCl, occasional addition of 2 *N* NaOH was sometimes necessary to maintain the *p*H at about 8. After some time the mixture was extracted with ether to remove unchanged mustard gas and the resulting solution used for immunization or for the serological tests. No attempt was made to separate the altered proteins from the mixture, since the sole object of early studies was to determine whether any change had occurred which could be detected immunologically.

Mustard gas-treated proteins. A mixture of 100 ml. of serum, 100 ml. of a phosphate buffer of *p*H 8·0 (100 ml. of 0·2 *M* KH_2PO_4 plus 93·6 ml. of 0·2 *N* NaOH) and 1 g. of NaCl was stirred at room temperature for 8 hr. on each of three successive days, after the addition of 2 ml. of mustard gas on the first and second days. During each night the mixture was kept in the ice-chest. It was finally extracted five times with 200 ml. of ether each time, the ether removed by evacuation and the *p*H adjusted to 7·5. The preparations used for immunization were treated with 0·05 vol. 5 % phenol.

"Sulphone"-treated proteins. These were prepared in the same way as were the mustard gas-treated proteins but the solid $\beta\beta'$-dichlorodiethylsulphone was added in ethereal solution (two additions of 2 g. of the sulphone in about 45 ml. of ether to 100 ml. of serum). In other experiments the reaction was carried out at 30° instead of at room temperature, and the two preparations are referred to in this paper as sulphone-treated proteins (17°) and sulphone-treated proteins (30°).

Immunization. The antigens injected were all prepared from normal horse serum. The method of immunization was that recorded previously [Hopkins & Wormall, 1933, 1], all the injections being made intraperitoneally. The injected rabbits were bled after 3–7 injections. The serological reactions mentioned below were often observed after the third injection but a better response was obtained after six or seven injections.

Precipitin tests. These tests were carried out as described by Hopkins & Wormall [1933, 1].

Complement-fixation tests. Mixtures of 0·5 ml. of diluted antigen, 0·10 ml. of the antiserum (previously heated at 56° for 20 min.) and 0·10 ml. of 1:5 guinea-pig serum (in 0·9 % NaCl) were kept at room temperature for 1 hr. To each tube was then added 0·5 ml. of a 4 % suspension of sensitized ox red cells (in 0·9 % NaCl) and the tubes were immersed in a water bath at 37°. Readings were taken at various intervals. The results given in Table II are typical results obtained after 1 hr.

Results of the serological tests

The injection of mustard gas-treated horse serum into rabbits results in the production of antibodies which give precipitin reactions with the antigen injected and to a lesser extent with mustard gas-treated proteins of rabbit serum (or chicken serum). The reactions observed (Table I) were sufficiently pronounced to establish definitely that a new specificity had been acquired. The retention of the original species specificity of the serum proteins, shown by the reactions between horse serum and antisera to mustard gas-treated horse serum and between mustard gas-treated horse serum and antisera to horse serum, is not unexpected since the mustard gas-treated protein preparations undoubtedly contained unchanged serum proteins. In view of the possibility that the precipitin reactions with the mustard gas-treated proteins might be related to groups substituted in the free amino-groups of the protein, cross-tests were made with phenylcarbamido-proteins (proteins in which the free amino-groups have been replaced by $NH.CO.NH.C_6H_5$). No cross-reactions were observed, and this is perhaps not surprising in view of the sharp specificity observed when new groups are introduced into protein molecules (for a review of the literature cf. Landsteiner [1936]; Marrack [1938]). Confirmation of the reactions between antisera to mustard gas-treated horse serum and mustard gas-treated rabbit serum was obtained by complement-fixation tests, representative results of which are given in Table II.

Table I. *Precipitin reactions with antisera to mustard gas-treated proteins*

		Immune serum against				
		Mustard gas-treated horse serum			Horse serum	Phenyl-carbamido-horse serum proteins
Antigen	Dilution of antigen	No. 498	No. 198	No. 199	No. 82	No. 100
Horse serum	1:20	+	±	tr.	+ +	–
	1:100	+ +	+	±	+ +	–
	1:500	+ ±	+	±	+ +	–
	1:2500	+ ±	±	±	±	–
Mustard gas-treated horse serum	1:20	+ +	+ + ±	+ ±	+ +	–
	1:100	+ + +	+ + ±	+ ±	+ +	–
	1:500	+ +	+ ±	±	+ +	–
	1:2500	±	±	±	±	–
Mustard gas-treated rabbit serum	1:20	–	–	–	–	–
	1:100	±	±	±	–	–
	1:500	±	+	+	–	–
	1:2500	tr.	±	tr.	–	–
Mustard gas-treated chicken serum	1:20	–	–	–	–	–
	1:100	–	f. tr.	f. tr.	–	–
	1:500	±	±	±	–	–
	1:2500	f. tr.	f. tr.	f. tr.	–	–

The dilutions of antigen in Tables I, II and III refer to dilution of a solution which contains 5% of total protein. Precipitin test readings (taken after 1 hr. at 37°): – (no reaction), f. tr. (faint trace), tr. (trace), ±, +, + ±, + + etc., in increasing degrees of precipitation.

Similar results were obtained with the proteins treated with $\beta\beta'$-dichlorodiethylsulphone. Precipitin tests (Table III) and complement-fixation tests showed that antisera to the sulphone-treated proteins are capable of reacting with sulphone-treated rabbit serum; they will not react, however, with mustard gas-treated rabbit serum. In these experiments the best reactions were given with the preparations obtained by allowing the sulphone to act on the proteins at 30°,

Table II. *Complement-fixation tests*

Antigen dilution	Antigen: Immune serum	Mustard gas-treated horse serum: No. 498	Mustard gas-treated horse serum: No. 198	Mustard gas-treated rabbit serum: No. 498	Mustard gas-treated rabbit serum: No. 198
1:20		3	2	4	4
1:60		1	1	3	4
1:180		—	—	3	2
1:540		—	—	2	—
1:1,620		—	—	1	—
1:4,860		—	—	—	—
1:14,580		—	—	—	—
1:43,740		—	—	—	—
1:131,220		—	1	1	—
1:393,660		1	3	2	1
1:1,180,980		2	4	4	2
1:3,542,940		4	4	4	4
Control (NaCl)		4	4	4	4

Readings: 4 (complete haemolysis); — (no haemolysis); 3, 2 and 1 intermediate degrees of haemolysis.

Antisera: Nos. 498 and 198 (antisera to mustard gas-treated horse serum).

and its seems probable that at body temperature the chemical reaction between proteins and mustard gas (or the corresponding sulphone) may be appreciably quicker than that observed in the experiments recorded here. Further experiments are now being carried out to determine the minimum time required for mustard gas and the sulphone to produce at 37° a change in the protein molecule which can be detected by these serological methods.

Table III. *Precipitin reactions of antisera to "sulphone"-treated proteins*

Antigen	Dilution of antigen	Immune serum against: Sulphone-treated horse serum: No. 403	Sulphone-treated horse serum: No. 404	Sulphone-treated horse serum: No. 406	Sulphone-treated horse serum: No. 408	Mustard gas-treated horse serum: No. 493
Horse serum	1:20	tr.	tr.	–	–	tr.
	1:100	±	±	±	tr.	+
	1:500	tr.	tr.	tr.	tr.	±
	1:2500	–	tr.	f. tr.	–	tr.
Sulphone-treated horse serum (30°)	1:20	+ +	+ + ±	±	+	f. tr.
	1:100	+ +	+ + ±	+	+	±
	1:500	+	+ ±	+	+	+
	1:2500	tr.	±	tr.	tr.	tr.
Sulphone-treated rabbit serum (30°)	1:20	–	tr.	–	–	–
	1:100	tr.	+	f. tr.	f. tr.	–
	1:500	+	+	f. tr.	tr.	–
	1:2500	+	+	tr.	±	–

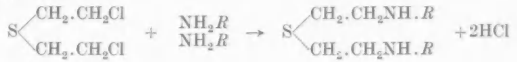
Immune sera nos. 403 and 404 were obtained with the sulphone-treated horse serum (30°) and nos. 406 and 408 with sulphone-treated horse serum (17°).

Discussion

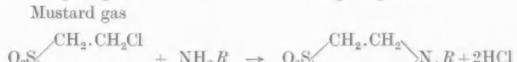
The results of the experiments described here show that mustard gas and $\beta\beta'$-dichlorodiethylsulphone effect a change in proteins which can be detected by immunological methods. The change is sufficient to convert the protein into derivatives which may be considered "foreign" to the homologous animal. Some of the more characteristic features of the vesicant action of mustard gas are the smallness of the effective dose, the delayed action and the fact that people who have been badly "gassed" are liable to become hypersusceptible to mustard gas.

These facts are compatible with the view that the biological mechanism of the reaction may be related to protein sensitivity, but whether the present results have any bearing on this problem cannot be decided without further investigation.

The identity of the chemical changes effected in the protein molecule by mustard gas and the sulphone has not yet been established, but it seems probable that they are concerned with the free amino-groups of the protein. Cashmore & McCombie [1923], using glycine ester, obtained products which suggest reactions of the following types:



Mustard gas



Lawson & Reid [1925] obtained similar results but found that although amino-acids condense readily with the sulphone they do not react with the sulphide (mustard gas). In these experiments of Cashmore & McCombie and those of Lawson & Reid, the conditions of the reaction were much more drastic than those employed by us. In the investigations described here, however, it has been necessary to avoid high temperatures and the use of strong alkalis or acids, in order that the antigenic power of the proteins might not be destroyed. Furthermore, it was the intention to reproduce as far as possible reactions which might take place when mustard gas is allowed to come into contact with the animal body. In spite of these differences it seems probable that the protein changes detected by the serological tests are similar to those occurring in amino-acids and their esters. The differences between the actions of mustard gas and of $\beta\beta'$-dichlorodiethylsulphone [Cashmore & McCombie, 1923] would readily explain the failure, in our experiments, of proteins treated with mustard gas and those with the sulphone to give serological cross-reactions (cf. Table III).

In connexion with these studies of the action of mustard gas on proteins there are several other observations which appear to be pertinent. Arising out of the work on the anticarcinogenic action of mustard gas [Berenblum, 1935], Berenblum *et al.* [1936] studied the effects of mustard gas and $\beta\beta'$-dichlorodiethylsulphone on the respiration and glycolysis of minced Jensen sarcoma tissue *in vitro*. They found that these compounds reduce the glycolysis of the tumour material more than its respiration. Jány & Sellei [1935] also noted that mustard gas inhibits glycolysis by *B. coli* and by Ehrlich rat sarcoma. Peters [1936] has shown that $\beta\beta'$-dichlorodiethylsulphone has an inhibitory effect, similar to that of iodoacetic acid, on the respiration of brain tissues; this inhibition is exercised on the change pyruvate → oxidation products, and the sulphone has no appreciable effect on the conversion, in this tissue, of lactate into pyruvate. The inactivation of certain enzymes in the tissues studied by these various authors may possibly be due to the same change which produces an alteration in the immunological properties of the protein. Investigation from both aspects may, therefore, throw some light on the nature of the action of mustard gas and related compounds on the animal body.

Summary

1. $\beta\beta'$-Dichlorodiethylsulphide (mustard gas), when allowed to act on horse-serum proteins at room temperature, produces a change in the immunological properties of these proteins. These derivatives produce in the rabbit antibodies which are capable of reacting with mustard gas-treated rabbit serum.

2. $\beta\beta'$-Dichlorodiethylsulphone-treated horse-serum proteins produce antibodies which will react with sulphone-treated serum proteins, but not with the mustard gas-treated proteins.

3. The possible chemical basis of this change in immunological properties may be an interaction between the mustard gas (or sulphone) and the free amino-groups of the protein molecule.

The authors would like to take this opportunity of thanking the Medical Research Council for a grant to one of them (A. W.) to cover part of the expenses incurred in this work.

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XI. STUDIES ON BAYER 205 (GERMANIN) AND ANTRYPOL[1]

III. FURTHER OBSERVATIONS ON THE METHOD OF DETERMINATION AND ON THE RETENTION OF THIS DRUG IN THE ANIMAL BODY

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In a previous communication [Dangerfield *et al.* 1938] a method for the determination of Bayer 205 and Antrypol in the blood plasma was described, and experimental evidence was produced to show that after the injection of a "normal" dose of Bayer 205 into a rabbit or dog there is a significant amount of the drug in the plasma 3 months or even longer after the injection. Further investigations have been concerned (*a*) with the simplification of the method (by the use of a Lovibond comparator) so that Bayer 205 determinations on blood plasma, C.S.F. and possibly other body fluids might be possible in tropical countries when a colorimeter is not available, and (*b*) with a modification of the method for determinations on urine. The observations on the retention of the drug in the body have also been extended in order to provide a continuous curve to show the amount of Bayer 205 in the plasma of rabbits during and after a series of injections of the drug. During these investigations no appreciable difference has been observed between Bayer 205 and Antrypol, and in most of the later experiments the latter preparation has been used.

Method of determination

It has not been found necessary to make any significant alteration in the method described in the earlier paper, but one or two slight improvements have been introduced. The hydrolysis by conc. HCl is now effected in glass-stoppered pyrex tubes graduated at the 10 ml. level. At one period in these investigations difficulty was experienced in matching the final colours, owing to occasional development of turbidity following the addition of the methyl-α-naphthylamine solution. It was found that this turbidity was due to impurities (possibly Cu) in the distilled water, and this trouble was overcome by passing the distilled water twice through a permutit water-softener.

Subsequent work has fully confirmed the suggestion that methyl-α-naphthylamine hydrochloride has many advantages over the free base when used as the coupling agent. The free base (an oil) develops a red colour fairly rapidly whereas the solid hydrochloride shows very little change in colour over a long period, particularly if exposure to sunlight is reduced to a minimum. The solution used in the actual determination (0·2 g. in 100 ml. in 50% v./v. acetic acid) shows very little reddening in 1 or 2 weeks if kept in a dark bottle.

[1] This communication represents a second report to the Medical Research Council at whose request this work was undertaken.

Preparation of methyl-α-naphthylamine hydrochloride. The preparation of the free base was essentially that described by Fischer [1895]. The following additional details of the method might prove useful. α-Naphthylamine was formylated [Tobias, 1882], and the product recrystallized and then methylated. For the methylation the xylene should be perfectly dry and granulated sodium should be used. After the addition of the sodium, a vigorous reaction occurred and a white precipitate formed; the mixture was cooled to 80°, methyl iodide added and the temperature raised to 100–110°, with subsequent completion of the reaction by boiling the mixture under reflux for 1 hr. The cooled product was filtered and the filtrate was treated with charcoal and freed from xylene by steam distillation. The formylmethyl-α-naphthylamine was hydrolysed by boiling for 1 hr. under reflux with 5 vol. dil. H_2SO_4 (10% v./v.), the solution filtered and treated with charcoal, and the final filtrate kept overnight, when a little α-naphthylamine separated out. The filtered solution was basified with NaOH, and the base extracted with ether. The ethereal solution was treated with charcoal and dried with $CaCl_2$. Dry HCl was passed into this solution to precipitate methyl-α-naphthylamine hydrochloride. The precipitate obtained in the early stages contained some violet impurity and was discarded. The main bulk precipitated by further passage of HCl was dried *in vacuo* in the presence of solid KOH. No further purification by recrystallization was possible but this preparation, which had only a very faint pink tinge, was pure enough for the purposes of this investigation. The yield was 12·8 g. of the hydrochloride from 14 g. of crude base, and a similar yield was obtained from a sample of the base supplied by Messrs May and Baker, Ltd., Dagenham.

The use of a Lovibond comparator. At the suggestion of Dr F. Hawking, who wished to carry out determinations of Bayer 205 in East Africa, coloured discs have been made for this purpose by "The Tintometer Ltd." A set of two discs covers the ranges equivalent to 0·18 to 1·20 and 1·20 to 3·60 mg. of hydrolysed Bayer 205 per 100 ml. of solution to be diazotized. The matching of the colours is quite easy and can be done with accuracy up to the reading of 2·70. With larger amounts of Bayer 205 it is advisable to use a smaller volume (i.e. less than 2 ml.) of the hydrolysed solution and to add 3 *N* HCl to give 2 ml. for the diazotization process. When these discs are used it is possible to determine with satisfactory accuracy amounts of Bayer 205 greater than 0·5 mg./100 ml. of plasma or other fluid. On the rare occasions when smaller amounts have to be determined, the solution should be compared with a set of standards.

The comparator method has several advantages over the colorimeter method. Normal plasma and sera contain a small amount of diazotizable substances which can easily be allowed for by the use of the control tube, and this compensation for "blank values" has proved of considerable value for the determination of Bayer 205 in urine and certain animal tissues. When a "blank" of this type is not available, compensation for any yellow or brownish colour in the filtered hydrolysates can be made by using, as a control, a mixture of the filtrate and all the other reagents except the sodium nitrite.

Products of acid hydrolysis of Bayer 205

Lang [1931] states that the hydrolysis of Bayer 205 by hot HCl yields *m*-aminobenzoic acid, *m*-amino-*p*-toluic acid and α-naphthylamine-4:6:8-trisulphonic acid, but there appears to be no evidence that the disruption of the complex molecule of the drug is as complete as this. For another aspect of our investigations these three amines are required, and the opportunity has been taken to determine whether a mixture of the three would give the same colour

when diazotized and coupled with methylnaphthylamine as does an equivalent amount of hydrolysed Bayer 205. The colour obtained with an equimolecular mixture of these amines was approximately as intense as, but definitely more purple than, the colour with hydrolysed Bayer 205. The results suggest that a large part of the colour with the last-named is due to *m*-aminobenzoic acid. Each of the other two amines gives a lilac colour and these observations tend to show that hydrolysis with HCl is not as complete or simple as was suggested by Lang. This point is perhaps of theoretical interest only at present, and does not affect in any way the method of determination of Bayer 205, but it may be of significance when the possible decomposition of this drug in the body is studied.

Determination on serum

For some purposes it may be necessary to use serum for these determinations, and a comparison has been made of the values obtained with the plasma and sera of rabbits which had previously received injections of Antrypol. Blood was collected from the ear of each rabbit in amounts of 2·5, 5·0 and 2·5 ml. The first and third samples were collected in oxalated tubes and were then mixed and centrifuged for the separation of the plasma. The second blood sample was allowed to clot and the serum obtained. Bayer 205 determinations were made, and the results recorded in Table I show that the plasma and serum from blood containing Bayer 205 contain the same amount of this drug.

Table I. *Bayer 205 (or Antrypol) determinations on plasma and serum*

	mg. Antrypol per 100 ml.	
	Plasma	Serum
Rabbit 493 (Antrypol)	3·50	3·60
,, 494 (Antrypol)	3·40	3·40
,, 495 (Control)	0·95	1·00
,, 496 (Control)	0·95	0·95

The results in this table have not been corrected for the blank value of approximately 1·0 mg./100 ml. for normal rabbit plasma or serum.

Determinations on urine

In an earlier paper [Dangerfield *et al.* 1938] it was mentioned that the method devised for the determination of Bayer 205 in plasma cannot be used for determinations of the drug in urine because of the presence in normal urine of a very variable amount of diazotizable substances. The necessary blank values may be as low as 0·25 or as high as 5·0 mg./100 ml. (calculated as hydrolysed Bayer 205), and it is not possible by any simple method to compensate for this blank value. There is no satisfactory evidence as yet that unchanged Bayer 205 is excreted in the urine, but the excretion in the urine of a trypanocidal agent after the injection of the drug [Mayer & Menk, 1921; 1922; Thomson & Robertson, 1922] and some preliminary observations recorded earlier in the present investigations [Dangerfield *et al.* 1938] suggest that this excretion does take place. Further investigations which will be reported later leave no doubt that Bayer 205 either in the original or slightly modified form, is excreted in the urine in fairly appreciable amounts for a few days after the injection.

Since the use of a blank value for normal urine is precluded by the wide variation in this value, efforts have been made to destroy the "normal" amines without loss of Bayer 205, but without success. Ultimately it was found possible, however, to separate these normal amines (or "amine-precursors") from Bayer

205 by ultrafiltration through a standard collodion membrane, and this is the basis of the method now adopted. Collodion membranes are prepared by the method described by Folley [1933] and are dried for 15 min. in a current of air passing though the apparatus at the rate of 2 l. per hr. at room temperature (19–21°). The membranes are then immersed in distilled water for 24 hr., and filtration is effected with a positive pressure of 120 mm. Hg in the apparatus described by Folley. Normal urine (of man or dog) yields an ultrafiltrate which, on hydrolysis with HCl, gives the same amount of diazotizable amine as does the untreated urine, but no Bayer 205 passes through the membranes. If determinations are made, therefore, on a urine containing Bayer 205 and on an ultrafiltrate from this urine, the difference between the two values is a measure of the Bayer 205 present.

The "recovery" of Bayer 205 when added to urine is, however, not quantitative, but usually varies between 70 and 95 % with an average of about 85 %. (With one sample of urine, however, the recovery was a little below 50 %.) This loss is fairly constant for any given sample of urine, e.g. with the same urine the loss may be 15 % with amounts of Bayer 205 varying from 1 to 10 mg./100 ml. (cf. Table II). A fairly satisfactory estimate of the true Bayer 205 content of the urine can thus be obtained by the use of a Bayer 205 "recovery factor" for the urine concerned, this factor usually being between 1·10 and 1·25. For a true estimate of the amount of Bayer 205 in any sample of urine it is necessary therefore, to carry out determinations on (*a*) the urine, (*b*) an ultrafiltrate from the urine, prepared as described above and (*c*) the urine treated with a known amount of Bayer 205 (e.g. 2 or 3 mg./100 ml. of urine). If (*c*) is prepared by the addition of a small amount of strong Bayer 205 solution to the urine, the urine for (*a*) and (*b*) should be diluted with a corresponding amount of water. The difference between (*a*) and (*b*) will give the minimum Bayer 205 content of the urine (or diluted urine), and a more correct value is obtained by multiplying this value by a factor if the difference between (*a*) and (*c*) shows that the recovery is not complete. With the Lovibond comparator method these differences can be measured very readily since the less-coloured sample can be placed in the control tube. (A control determination is, of course, made on the Bayer 205 solution used for the preparation of (*c*), and in all the experiments quoted here duplicate determinations were made.)

Several possible explanations for this incomplete recovery of Bayer 205 added to urine have been examined, in the hope that some slight modification might render the use of a recovery factor unnecessary. A longer period of hydrolysis (8 hr. instead of 6 hr.) or an increase in the amount of HCl, does not increase the yield. One of the factors concerned is possibly the presence in hydrolysed urine of substances which prevent the maximum colour development, e.g. the liberation of phenolic substances from ethereal sulphates and the combination of these phenols with the diazotized amines. The addition of hydrolysed urine to a separately hydrolysed Bayer 205 solution causes only a slight loss of amine (about 5 %), and this explanation can account for only part of the total loss. It was ultimately decided that it is wiser to adopt a correction factor in these investigations rather than attempt to devise a complicated technique which may lead to greater inaccuracies.

Some of the results of determinations on normal urines after the addition of Bayer 205 are recorded in Tables II and III, and from these results it will be seen that the method can be used for the detection and determination of small amounts of Bayer 205 or Antrypol. Amounts greater than 0·6 mg./100 ml. of urine can be determined with satisfactory accuracy. A chemical method of this type cannot,

Table II. *Recovery of Bayer 205 added to normal urine*

Subject	Amount of Bayer 205 added	"Amine" plus Bayer 205	Bayer 205	Recovery %
	mg. Bayer 205/100 ml.			
1	0	0·66	—	—
	1·0	1·48	0·82	82
	2·0	2·33	1·67	83
	3·0	3·07	2·41	80
	5·0	4·67	4·01	80
2	0	1·93	—	—
	0·97	2·66	0·73	75
	3·10	4·33	2·40	77
3	0	0·70	—	—
	0·95	1·60	0·90	95
	3·0	3·27	2·57	86
	11·0	10·0	9·30	85
4	0	0·77	—	—
	1·0	1·59	0·82	82
	3·2	3·33	2·56	80
	10·7	10·20	9·43	88
5	0	0·67	—	—
	1·00	1·60	0·93	93
	3·0	3·43	2·76	92
	10·3	10·40	9·73	94
6	0	0·73	—	—
	1·00	1·20	0·47	47
	2·20	1·62	0·89	40
	4·50	2·68	1·95	43
	7·85	4·13	3·40	43

The above results are average values of two or three determinations (i.e. separate hydrolyses). The urines for these determinations and for those given in Table III were diluted with one-ninth their volume of water or Bayer 205 solution (of ten times the strength recorded in column 2).

Table III. *Determination of Bayer 205 (or Antrypol) in urine*

Amount of Bayer 205 added to urine	Bayer 205 plus "amine"		Bayer 205 value	Corrected* Bayer 205 value
	Untreated urine	Urine ultrafiltrate		
2·0	2·59	0·98	1·61	2·07
0	1·61	1·60	—	—
1·0	2·47	1·55	0·92	1·13
3·0	4·00	1·57	2·43	2·99
0	1·80	1·78	—	—
1·0	2·50	1·72	0·78	1·08
3·0	4·00	1·80	2·20	3·04
0	0·25	0·25	—	—
1·86	1·80	0·25	1·55	1·89
3·95	3·33	0·25	3·08	3·76
5·87	5·08	0·25	4·83	5·89
0	0·72	0·70	—	—
2·17	2·59	0·72	1·87	2·19
4·43	4·60	0·70	3·90	4·56
6·67	6·40	0·72	5·68	6·65

* Obtained by multiplying the figure in the previous column by a factor which is calculated from values obtained with mixtures of the untreated urine and known amounts of Bayer 205. This factor is usually between 1·10 and 1·25.

All the figures in the above Table represent mg./100 ml.

of course, differentiate between Bayer 205 and similar substances which might be produced from this drug in the animal body. The use of this ultrafiltration method for urine probably ensures, however, that relatively simple substances produced from Bayer 205 will not be estimated with the parent substance since the membranes used are completely permeable to *m*-aminobenzoic acid. *m*-Amino-*p*-toluic acid and α-naphthylamine-4:6:8-trisulphonic acid, the other amines which, according to Lang [1931], are produced by acid hydrolysis of Bayer 205, are retained by the membranes to a slight extent only.

Retention of Bayer 205 in the plasma following injection of the drug

A preliminary investigation to determine the length of time during which injected Bayer 205 could be detected in the plasma of rabbits following a single or a series of injections of the drug was described in an earlier paper [Dangerfield *et al.* 1938]. The results obtained suggested that a series of injections of small amounts results in a more satisfactory maintenance of a significant Bayer 205 level (above 1 mg./100 ml. of plasma) than does a single injection of a much larger amount. Since this problem is of considerable interest to those who are concerned with the prophylactic use of the drug in tropical countries, it was decided to extend these observations in order to obtain continuous curves to show the amount of Bayer 205 in the plasma of rabbits following (*a*) a large dose of the drug (equivalent to 6·3 g. for a 70 kg. man), and (*b*) three injections of one-third this amount, the three injections being made at intervals of about 5 weeks.

Experimental details. Three rabbits (group A) each received an intravenous injection of 0·09 g. Bayer 205/kg., and three others (group B) received 0·03 g./kg. Further injections, each of 0·03 g./kg., were given to the second group 36 days and 73 days after the first injection.

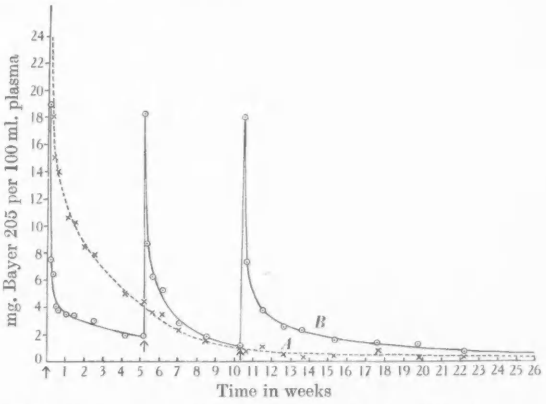

Fig. 1. Bayer 205 in the plasma of rabbits following the injection of the drug. ×---× Group A (one injection). o—o Group B (three injections). The arrows indicate when the injections were made.

Blood samples (about 6 ml.) were withdrawn from the marginal vein of the ear before, and 3 and 24 hr. after, each injection and at suitable intervals during the remaining periods. The blood was oxalated and centrifuged immediately. The amount of Bayer 205 in the plasma was then determined as previously

described [Dangerfield *et al.* 1938]. The determinations were made with a colorimeter and in most cases by the comparator method described in this paper; no significant difference between the two results was observed. Determinations of the "blank value" for normal rabbits were made at frequent intervals. These proved to be practically constant at the equivalent of approximately 1 mg. of Bayer 205/100 ml. of plasma and all values for the experimental animals have been corrected accordingly.

The results of this experiment are recorded in Fig. 1, the values recorded representing averages for the two groups of rabbits.

Discussion

Further study has shown that the previously described method of determination of Bayer 205 (or Antrypol) in plasma [Dangerfield *et al.* 1938] presents few difficulties and is satisfactory for the determination of amounts of the drug which are likely to be significant. No serious modification of the method has been found necessary, but the use of a Lovibond comparator with appropriate discs renders the determination much easier and makes it possible for measurements to be made when a colorimeter is not available. Throughout the whole of this work the authors have kept in mind the probability that workers in tropical countries, carrying out investigations under somewhat difficult conditions, might wish to make Bayer 205 determinations. For this reason, the observation made here, that serum and plasma give the same values, will be of special interest. Further experiments are now being made to determine the best method of preserving serum which will allow accurate determinations to be made several days or even weeks after the blood has been collected.

A considerable amount of time has been devoted to rendering the method applicable to urine, for such determinations will have to be made before a true picture of the behaviour of Bayer 205 in the body can be obtained. Although there is little or no chemical evidence that the drug is excreted in the urine after its injection into an animal, there is no doubt that this excretion does occur. The investigations on man now being carried out, involving an attempt to trace the fate of most of the Bayer 205 injected, necessitate measurement of the excretion, and the method described in this paper appears to be quite satisfactory for this purpose. The method is, unfortunately, more complicated than that for plasma or serum, but there is as yet no indication that urinary Bayer 205 determinations on patients receiving this drug will normally be needed. The more important value, as far as treatment is concerned, is almost certainly the plasma Bayer 205 level. If it is found necessary to make determinations on urine, the latter can be preserved by the addition of toluene, and the hydrolyses and determinations made at a later date.

The results of the experiment on rabbits confirm the suggestion made previously that for the maintenance of a significant level of Bayer 205 in the plasma it is better to have a series of injections rather than one massive dose (cf. Fig. 1). No evidence has yet been obtained that the latter builds up a higher reserve in the body, and from other evidence available it seems probable that a considerable part of the extra Bayer 205 injected in the large dose is excreted in the urine during the first few days. The amount of Bayer 205 or Antrypol usually injected into man for the curing or prevention of sleeping sickness varies very considerably [cf. Findlay, 1930, pp. 262–8], but the customary dose is 1 or 2 g. for the average man. These injections are often repeated at intervals of a few days or every week, and the total amount injected over a period of 6 months may be 5 g. or even as much as 7–10 g.

The long retention of Bayer 205 in the animal body is remarkable. It seems probable that the persistence of Bayer 205 in the plasma and tissues accounts for the very satisfactory prophylactic value of this drug as far as sleeping sickness is concerned. Information which would be very useful in this connexion can perhaps be classified as follows: (i) the minimum plasma–Bayer 205 level which will normally protect against sleeping sickness when the individual is exposed to infected tsetse flies, (ii) the best method of ensuring that this level will be exceeded without excessive administration of the drug, and (iii) the simplest way of making Bayer 205 determinations on the plasma of patients. The method described in this paper is suitable for the last-named determinations in ordinary field laboratories, but the other questions cannot be dealt with so readily. The minimum "protective" Bayer 205 level for man can best be established by numerous determinations on patients who develop the disease after treatment with the drug. In that way it may be possible to say that if the amount of Bayer 205 in the plasma falls below a certain value the protection against trypanosomiasis is not adequate. Another method, and the one which the authors propose to adopt, involves experiments on small animals (rabbits, mice etc.), since the natural conditions (man as the experimental animal and infection by the bite of infected tsetse) cannot be reproduced readily in this country.

The best method of maintaining a protective level of Bayer 205 in the plasma can only be satisfactorily established when the average minimum protective level has been fixed, but for present purposes it might be assumed (very tentatively) that values above 1 or 1·5 mg./100 ml. of plasma may be significant under normal conditions. If this is not so, the protective effect observed in investigations on volunteers injected with Bayer 205 and subsequently exposed to the bites of infected tsetse flies [Duke, 1934] and the almost universal agreement that small amounts of the drug will give protection for several weeks [cf. review of literature by Findlay, 1930] are difficult to explain; otherwise it would be necessary to assume that the protective action is not directly related to the Bayer 205 present in the plasma and in tissue fluid. In the light of these chemical studies, therefore, there does not appear to be justification for either a very large dose of the drug (greater than 2 g. for an average man) or for several injections at very short intervals (e.g. every few days). A series of three injections made every 4 or 5 weeks should serve to maintain the plasma-Bayer 205 level above 1 mg./100 ml. for a total period of 4 or 5 months. It is not suggested however that the valuable clinical observations of those who prefer to give several injections over a period of 1 or 2 weeks should be disregarded, for until a "minimum protective level" (if such a value does exist) can be determined, it would be undesirable to contrast critically the various methods of prophylactic treatment.

The possibility that Bayer 205 is stored in certain parts of the body is being studied at the present time. These experiments are as yet incomplete, but there seems to be no doubt that the drug is not stored to any significant extent in organs such as the liver, spleen etc. Other experiments, which will be described in a later paper, show that some type of combination occurs between Bayer 205 and plasma proteins, and the long retention of the drug in the animal body is most probably due to its combination with plasma and tissue proteins.

Summary

1. Further observations have been made on the method of determination of Bayer 205 (or Antrypol) previously described [Dangerfield *et al.* 1938]. The technique has been simplified by the preparation of coloured discs for a Lovibond comparator.

2. Plasma and serum obtained from a Bayer 205-containing blood contain the same amount of the drug.

3. A modification of the method has been devised for the determination of small amounts of Bayer 205 in urine. The difference between the values for the urine and an ultrafiltrate obtained by filtration through a standard collodion membrane is a measure of the Bayer 205 present. This difference gives a minimum value which can be rendered more accurate by the use of a correction value. This factor is usually between 1·10 and 1·25, and it can be determined quite easily.

4. Investigations have been carried out which support the suggestion made in a previous paper that a course of injections of the drug results in a more satisfactory maintenance of a significant amount of Bayer 205 (or Antrypol) in the plasma over a long period than does a single injection of the same total amount.

5. Some possible reasons for the long retention of the drug in the plasma are tentatively discussed.

The authors would like to express their gratitude to the Medical Research Council for personal grants to two of them (W. G. D. and later J. C. B.), and for a grant which has, in part, covered the expenses of this investigation. To Mr G. S. Fawcett of "The Tintometer Ltd." we are indebted for help with the preparation of coloured discs for the Lovibond comparator, and thanks are tendered to Messrs B.D.H. for a supply of Antrypol.

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XII. EXPERIMENTS ON THE METABOLISM OF GLYCINE

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THE metabolism of glycine has been a subject of controversy for a long time. As the simplest amino-acid, it was chosen as one of the first objects of investigation. But in the course of research it was seen that glycine behaved differently in many ways from the higher members of the series and that biochemical and physiological effects easily demonstrated with the higher aliphatic amino-acids proved to be absent or inconsistent with glycine.

The deamination of an amino-acid is considered to be one of its chief metabolic changes, and it was this mechanism on which early research was carried out. As long as the latter was confined to experiments involving the metabolism of the whole body, the results obtained by different investigators seemed to agree in general that glycine when fed *per os* [Salkowski, 1880; Nencki & Schultzen, 1872; Friedmann, 1908; Ringer & Lusk, 1910] or injected into the animal was almost quantitatively deaminated, the equivalent amount of NH_3 or urea being excreted by the animal. More recent experiments of Kiech & Luck [1931], however, demonstrated the existence of a lag between amino-N disappearance as estimated from the N content of the minced carcass, and urea formation following glycine administration. They conclude that "calculations of the extent of amino-acid metabolism from the amount of urea excreted during the first 8 hr. following amino-acid administration are likely to be seriously incorrect". Contradictions also arose when attempts were made to localize the deaminating effect on glycine by perfusion experiments with liver. Salaskin [1898] was the first to report urea formation during perfusion of liver with blood containing added glycine. Fiske & Karsner [1914], who repeated the experiment with improved technique, could not confirm these findings, whereas Jansen [1915] obtained small amounts of urea after perfusion. It is conceivable that the long duration of the experiments used by some of these workers (4–5 hr.) may finally have caused decomposition of the urea. The fate of the 2-C skeleton remaining after the deamination of glycine is also controversial. Feeding experiments such as carried out by Ringer & Lusk [1910] on phloridzinized dogs demonstrated an increased carbohydrate excretion following glycine administration which Pflüger [1910] could not confirm. More recently the same negative result was reported by Wilson & Lewis [1930], whereas Butts & Dunn [1935] observed no glucogenetic effect in one case, but report a small increase in carbohydrate content in another case.

Nor has agreement been reached hitherto either by means of the slice technique or by the use of tissue extracts. After Neubauer [1909] had discovered the principle of oxidative deamination in phenyl-substituted amino-acids. Patey & Holmes [1929] observed NH_3 production in chopped kidney tissue with added glycine. Krebs [1933] observed a slight deamination of glycine following its incubation with kidney and liver slices, but practically no effect was observed with kidney extracts, although a great number of other amino-acids were readily deaminated. Later Krebs [1935, 1], Bernheim & Bernheim [1935] and Kisch [1936],

confirmed the stability of glycine to deamination and oxidation, and Neber [1936] showed that of all the amino-acids tested glycine alone resisted deamination by intestinal mucous membrane. Reid [1936] obtained an increase in liver glycogen after injection with alanine, but a decrease following injection of glycine. Finally, similar results were experienced by Bach & Holmes [1937] when investigating the glucogenetic effect of amino-acids following their incubation with liver slices. Glycine proved to be the only amino-acid tested which caused no formation of carbohydrate, in fact a carbohydrate disappearance.

Thus it appears that glycine is probably metabolized by the body when fed to the whole organism, but the experimental conditions under which its deamination might consistently be demonstrated *in vitro* in different organs are not fully known. An attempt to elucidate such conditions and to clarify the mechanism of deamination seemed to be justified and represented the problem for this work.

Methods

Animals used. Young rats, chiefly albinos, all males with an average wt. of 200 g. normally fed on ordinary stock laboratory diet, were used for all experiments with surviving tissue slices and chopped organs. Cats were used for the perfusion experiments. Tissue extracts were prepared from organs of pig.

Method of perfusion. Two cats were used for each experiment, and ether was used as anaesthetic. One cat served as blood donator; from it about 140 ml. blood were drawn to which about 80 ml. blood of the second cat were added later. The blood was defibrinated, filtered and made up to 400 ml. with bicarbonate-Ringer solution. In the meantime a cannula was inserted into the hepatic portal vein of the liver of the second cat. When the kidney was perfused the cannula was inserted into the renal artery. The gall bladder was ligated in the case of liver perfusion. The respective organs were then removed from the animals, the blood of the second animal was treated in the same way as that of the first and added to the blood-Ringer solution. The Dale & Schuster apparatus was used for the perfusion with a flask oxygenator. Care was taken that the oxygenation was efficient. The blood colour changed from dark red after it had passed the organ to bright red after oxygenation. The speed of perfusion was such that the whole volume of the perfusion fluid circulated in the system within 7 min. Samples of the organs were clipped off at the beginning and at the end of the experiment and the cut surface was seared with a hot iron. The temp. was kept at 37° and it was observed that the perfusion fluid was evenly distributed throughout the organ perfused. The perfusion fluid circulated through the organ for 20 min. in absence of substrate. After that time glycine solution was added. Samples were taken at suitable intervals, the volume of each being recorded in order to control the volume of the remaining fluid. The duration of the perfusion varied in the different experiments from 90 to 150 min. At the end of the experiment the remaining perfusion fluid was emptied from the apparatus. The samples were immediately deproteinized by means of trichloroacetic acid, and NH_3- and NH_2-N estimations were carried out at once. For the isolation of glycine, $HgCl_2 + HCl$ was used as deproteinizing agent; the procedure of isolation is described below.

Experiments on surviving tissue slices and extracts. Technique of slicing was the same as described previously [Bach & Holmes, 1937]. The wt. of tissue slices was 100 mg. wet wt. in all exp. Slices could be conveniently cut from kidney, liver and brain. It was more difficult in the case of spleen, but it was possible to obtain sufficiently thin slices from this organ after some practice. The spleen slices then showed satisfactory respiration. The blood attached to the spleen

slices was not removed. Portions of the thinner parts of the diaphragm were used. Testis material was used without further treatment. The mucous membrane of the small intestine was used after washing the latter with water, cutting it lengthwise and scraping it off the intestinal wall. For the experiments with frog muscle an extract was prepared in the usual way. Heart tissue was cut in slices with scissors as it was not possible to slice it in the ordinary way with the razor. The tissue was in all experiments suspended in 3 ml. phosphate buffer *p*H 7·2, this volume including any added substrates.

Kidney extract was made from acetone powder [Keilin & Hartree, 1936] by suspending it in buffer solution, shaking it for 10 min. at room temperature followed by centrifuging. 2 ml. supernatant fluid, corresponding to 100 mg. acetone powder, were used for the experiment, 1 ml. buffer or substrate being added.

Barcroft cups and manometers were used. In the aerobic experiments the cups were evacuated and filled with O_2. In the anaerobic experiments the cups were 3 times evacuated and filled with N_2 passed over hot Cu filings. The exp. temp. was 38°.

The substrates after adjustment to *p*H 7·2 were in most cases added in dangling Keilin cups which were shaken off after equilibrium had been obtained. In the centre pot of the Barcroft cups was placed filter paper saturated with 0·3 ml. of 10 % KOH.

At the end of the experiment 2 ml. were drawn from the cups and made up to 10 ml. Aliquot portions of this fluid were used for NH_3 and urea estimations. Other portions used for estimations of total, amino-, amide- and purine-N, creatine and pyruvic acid, were deproteinized either with trichloroacetic acid (final concentration 6 %) or, in the later experiments with dialysed $Fe(OH)_3$ which gave more consistent results owing possibly to the avoidance of decomposiston of the glycine condensation products.

Estimation of NH_3 and urea. To the portion reserved for the NH_3 estimation 3 drops 3 % acetic acid were added, and NH_3 estimated after distillation [Parnas & Heller, 1924], collection in 0·01 *N* HCl and titration with 0·01 *N* NaOH. The portion of the fluid reserved for the urea estimation was incubated for 30 min. with urease at 60° followed by NH_3 estimation.

Estimation of NH_2-N. The deproteinized fluid was centrifuged, carefully neutralized and made up to 10 ml. To a suitable amount about 10 drops of $Mg(OH)_2$ suspension were added, the mixture placed on the boiling water bath for 25 min., the fluid being then acidified with 2 drops of glacial acetic acid. The estimation of NH_2-N was made with the Van Slyke apparatus.

Estimation of amide-N. To an aliquot portion of the deproteinized fluid H_2SO_4 was added to a final concentration of 5 %. It was placed on a boiling water bath for 30 min. after which it was neutralized and the NH_3 estimated.

Estimation of total N. Total N was estimated in a Kjeldahl apparatus, conc. NH_3-free H_2SO_4 with added Se and Cu salts being used for the incineration. The titration was carried out with 0·01 *N* HCl.

Estimation of purine-N. To 2 ml. deproteinized fluid 2 ml. 40 % $NaHSO_3$ and 2 ml. 10 % $CuSO_4$ were added. The reddish precipitate turned brown after 3 min. boiling. The fluid was centrifuged, the precipitate washed with 1 % acetic acid and centrifuged again followed by total N estimation on the precipitate [Krüger & Schmidt, 1905; Schmidt, 1933].

Estimation of creatine. A modification of Walpole's direct method [Walpole, 1911] of estimating creatine was used, diacetyl serving as reagent; it was prepared by distillation of dimethylglyoxime with H_2SO_4 [Walpole, 1911]. To 5 ml. experimental solution 0·5 ml. sat. Na_2CO_3 and 2 drops diacetyl were added.

The mixture was immediately placed in a boiling water bath for exactly 1 min. and then in cold water for 5 min. After a further period of 20 min. during which the samples were left standing at room temperature the red colour was measured as described below. Standard solutions with known amounts of creatine and controls with water were prepared in the same way. Since the control samples with water yielded a yellowish tint with the reagents alone, methods had to be developed to eliminate this interfering colour. Neither a colorimeter nor a tintometer served the purpose. In the later experiments the readings were carried out by means of a spectrophotometer. The light passing through the coloured solution of the water control was compared at different wave-lengths with that

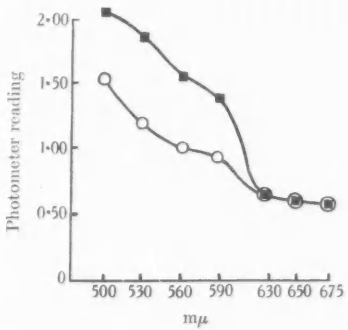

Fig. 1.

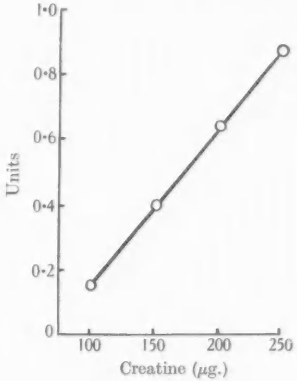

Fig. 2.

Fig. 1. Photometer readings. ■—■ With 100 g. creatine solution. o—o With the water control.

Fig. 2. Standard curve for creatine estimation. For definition of unit see text.

transmitted by known amounts of creatine. It will be seen from Fig. 1 that the two curves are almost contiguous down to 630 mμ (absorption = *b* arbitr. units), below which they show a divergence reaching a maximum value at approximately 530 mμ (absorption = *a* arbitr. units). The final standardizing curve was plotted by estimating *a* and *b* both for the water control and for mixtures containing different known amounts of creatine (*a'* and *b'*). A straight line was obtained when values of creatine in arbitrary absorption units from the equation: $(a'-b')_{\text{creatine}}$ minus $(a-b)_{\text{control}}$ were plotted against μg. creatine (Fig. 2). The influence of glycine on the colour developed during the creatine estimations was found to be negligible. That of glycocyamine was allowed for by adding this substance to the water control.

Estimation of pyruvic acid. The estimation of pyruvic acid was carried out on the deproteinized fluid according to the method of Clift & Cook [1932]. Special care was taken for reasons described that the excess $NaHSO_3$ was titrated with I_2 immediately in order to avoid possible decomposition of the product of condensation of glycine with pyruvate (see below).

Reagents. I have to thank Dr D. E. Green for a gift of cozymase solution and Mr E. M. Crook for that of coenzyme II. The other reagents were obtained from British Drug Houses, Ltd.

Experimental

In vitro experiments were first made on the deamination of glycine and *dl*-alanine under conditions in which many amino-acids besides alanine are readily deaminated. In all the cases in which NH_3-N was estimated, urea-N was determined at the same time, the total of both being given as "NH_3-N". The results from Table I show clearly that while alanine readily gives rise to NH_3 production, no such formation of NH_3 takes place under the same experimental conditions with glycine.

Table I. *NH_3 formation from glycine and* dl-*alanine in extracts of pig kidney*

Experimental period: 2 hr.

Kidney powder used for extract mg.	Amino-acid-N added mg.	mg. NH_3-N: Without added substrate	With added amino-acid	Extra NH_3-N formation	Extra NH_3-N formation as % of added NH_2-N
		Glycine			
100	0·56	0·14	0·16	0·02	0·4
200	1·12	0·21	0·12	−0·09	−8
100	1·12	0·05	0·05	0	0
100	1·12	0·08	0	−0·08	−7
50	0·56	0·07	0·04	−0·03	−5
250	1·12	0·14	0·14	0	0
*	1·12	0·08	0·08	0	0
		Alanine			
100	0·47	0·14	0·294	0·154	65†
200	0·94	0·21	0·39	0·18	38†
100	0·94	0·05	0·35	0·30	64†
100	0·94	0·08	0·35	0·27	57†
50	0·47	0·07	0·24	0·19	81†
250	0·94	0·14	0·42	0·28	60†
*	0·94	0·08	0·40	0·32	68†
				Average for alanine	62

* 1 g. rat kidney was ground with sand and buffer and centrifuged, the supernatant fluid being taken for the experiment.

† % disappearance is calculated from the amount of added *d*-alanine (i.e. ½ *dl*-alanine).

The same difference in behaviour between the two amino-acids was observed regarding the O_2 consumption. Fig. 3 gives the course of the respiration of kidney extract in presence of glycine + *dl*-alanine. It will be seen that while with alanine O_2 consumption reached the theoretical value calculated on the basis of oxidative deamination of the *d*-isomeride, glycine does not increase the O_2 consumption of the tissue at all. It is also shown that an addition of glycine + alanine does not affect the respiration as compared with that of alanine alone in tissue extracts. This phenomenon is equally shown by comparison of columns 4 and 6 in Table II in which the results of further respiration experiments are recorded. A comparison of columns 3 and 5 reveals a slight inhibition of O_2 consumption in presence of glycine alone.

Table III shows the effect of a mixture of glycine and alanine when incubated with various tissues. The object of these experiments was to investigate whether a "Stickland effect" such as observed in bacteria [Stickland, 1934; Woods, 1936] would affect NH_3 production. Results from Table III show that this is not the case. There is very little or no difference between the formation of NH_3, the disappearance of NH_2-N and the O_2 consumption in presence of alanine alone and those in presence of alanine plus glycine.

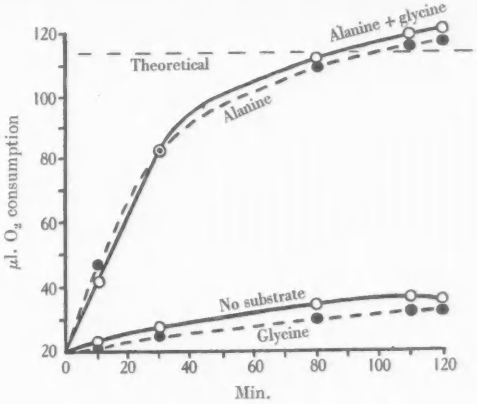

Fig. 3. Respiration of pig kidney extract with added *dl*-alanine and glycine.

Table II. *Respiration of kidney extract in presence of glycine and* dl-*alanine*

		μl. O_2 consumption			
Duration of exp. (min.)	Amino-acid conc. (%)	Without added substrate	With alanine	With glycine	With glycine and alanine
60	0·1	25	169	15	174
60	0·2	87	322	23	—
60	0·2	—	322	10	322
90	0·1	31	185	21	192
90	0·2	93	387	28	—
90	0·2	—	358	19	355

Table III. *Experiments with various tissues in presence of a mixture of glycine and* dl-*alanine*

				With added substrate		
Duration of exp. min.	Tissue	Substrate	Amino-acid-N added mg.	Extra NH_3-N formed mg.	Extra O_2 consumption μl.	% of NH_2-N disappeared mg.
60	Kidney extract	Glycine	0·56	−0·03	− 10	—
60		*dl*-Alanine	0·47	0·26	144	—
60		*dl*-Alanine + glycine	1·03	0·33	149	—
100	Kidney extract	Glycine	1·12	—	21	0
100		*dl*-Alanine	0·94	—	399	23
100		*dl*-Alanine + glycine	2·06	—	377	31
60	Kidney extract	Glycine	1·12	0·06	10	—
60		*dl*-Alanine	0·94	0·18	322	—
60		*dl*-Alanine + glycine	2·06	0·20	322	—
65	Kidney extract and spleen slices	Glycine	1·12	0	35	—
65		*dl*-Alanine	0·47	0·13	102	—
65		*dl*-Alanine + glycine	1·59	0·14	97	—

For further evidence determinations of NH_2-N were carried out, the results of which, shown in Table IV, seem to confirm the previous findings. While in the case of the alanine experiments an average of 82 % of the NH_2-N (calculated on

Table IV. *Disappearance of NH_2-N from glycine and* dl-*alanine in kidney extract*

Experimental period: 2 hr.

NH_2-N added mg.	mg. NH_2-N: Without substrate	With amino-acid added	Increase with amino-acid	Disappearance of added NH_2-N	Disappearance of NH_2-N in % of NH_2-N added
		Glycine			
1·12	0·47	1·50	1·03	0·09	8
1·12	0·47	1·82	1·35	−0·23	0
1·12	0·80	1·83	1·03	0·09	8
		dl-Alanine			
0·94	0·26	0·69	0·43	0·51	108*
0·47	0·26	0·50	0·24	0·23	100*
0·94	0·80	1·24	0·44	0·50	100*
0·94	0·47	1·14	0·67	0·27	58*
0·94	0·47	1·20	0·73	0·21	45*
				Average for alanine	82*

* Disappearance in % of added *d*-alanine-N (i.e. ½ *dl*-alanine).

the basis of the *d*-isomer) disappeared, practically all the glycine NH_2-N was present in most of the cases at the end of the experimental period. Another phenomenon observed was a discrepancy between the average disappearance of alanine-N of 82% and an NH_3-N formation of 62% (according to Table I). (Both series of determinations were carried out in the same experiment in order to obtain comparable values.) This phenomenon has previously been observed by Krebs [1933] and also by Neber [1936].

Table V. *NH_3 formation and O_2 consumption in tissue slices of various organs in presence of glycine*

Tissue wet wt. 100 mg. Duration 2 hr. Amount of glycine added 1·12 mg. glycine-N, except in exp. * in which it was 0·96 mg. Glycine conc.: 0·2% in all experiments.

Tissue	Extra NH_3-N with added glycine mg.	Glycine-N disappeared mg.	Extra O_2 consumption with added glycine, µl. after 60 min.
Rat kidney	0·04	—	−40
Rat heart	0·03	—	—
Rat heart	0	0·05	− 9
Rat spleen	0·05	—	−16
Rat spleen	−0·03	—	−12
Rat spleen	0·01	—	—
Rat intestine	0·01	—	—
Frog muscle	−0·04	—	0
Rat kidney	0·03	−0·10	−12
Rat heart	0·07	0·11	—
Rat kidney*	—	0·16	—
Rat kidney* 3·7 mg. glutathione	—	0·09	—
Rat kidney *p*H 7·8	—	0·07	—
Rat kidney *p*H 6·8	—	0·09	—
Rat testis	—	0	—
Rat brain	—	0·15	—
Rat spleen	—	0·12	—
Rat kidney	—	—	−61
Rat kidney	—	—	− 5
Pig kidney	—	—	− 5
Rat liver	—	—	14
Rat diaphragm	—	—	6

In the experiments recorded in Table V tissue extracts were replaced by tissue slices of various organs. None of the tissues used produced any appreciable amount of NH_3 or urea from glycine and none or little NH_2-N disappeared. In fact the respiration experiments revealed a slight but distinct inhibition in presence of glycine in almost all cases.

From the results obtained so far it seemed obvious that the deamination of glycine cannot be demonstrated by incubating it *in vitro* with either tissue extract or tissue slices under conditions such that with other amino-acids NH_3 or urea is readily formed and O_2 is taken up. Therefore perfusion experiments with added glycine on the intact organ were carried out as recorded in Tables VI and VII. The technique has been described earlier. NH_3- and NH_2-N estimations

Table VI. *Perfusion of cat liver*

Quantities: mg. N/100 ml. perfusion fluid or per g. liver.

	NH_3- and urea-N						
	In perfusion fluid			In perfused liver			Amount of glycine-N added (mg.)
Time course of perfusion I min.	NH_3-N	Urea-N	NH_3-plus urea-N	NH_3-N	Urea-N	NH_3-plus urea-N	
	Without added glycine						
0	0·4	4·6	5·0	0·18	0	0·18	0
20	0·4	4·1	4·5	0·18	0	0·18	0
	With added glycine						
25	0·28	5·57	5·85	—	—	—	55
80	0·63	8·17	8·80	—	—	—	55
110	1·12	9·28	10·40	0·12	0·35	0·47	55
Final result perfusion I	+0·72	+5·18	**+5·90**	−0·06	+0·35	**+0·29**	**55**
Final result perfusion II	+3·93	+1·33	**+5·36**	0	−0·38	**−0·38**	**96·5**
Final result perfusion III	−0·14	+3·68	**+3·54**	−0·03	−0·18	**−0·21**	**33·6**

Table VII. *Perfusion of cat kidney*

Quantities: mg. N/100 ml. perfusion fluid or per g. kidney.

	NH_3 and urea-N_2				
	In perfusion fluid			In perfused kidney	
Duration of perfusion min.	NH_3-N	Urea-N	NH_3-plus urea-N	NH_3-plus urea-N	NH_2-N in perfusion fluid
	Without added glycine				
0	0·28	7·00	7·28	0·71	10·5
15	0·28	—	—	—	7·35
	With added glycine				
75	0·70	—	—	—	—
105	0·28	5·60	5·88	0·77	43·7
Final result	0	−1·40	**−1·40**	**+0·06**	**36·35**
			Amount of glycine-N added		**34·3**

were performed both on the perfusion fluid and the organs used. Glycine was isolated from the former as the β-naphthalene-sulphonic compound.

In Table VI the course of a liver perfusion (I) has been briefly outlined whereas from liver perfusions (II) and (III) only the final balance is given for

simplicity's sake. Table VII shows the course of kidney perfusion and also includes NH_2-N determinations.

From the final results of Tables VI and VII it is evident that the NH_3 and urea formation observed during the perfusion is insignificant, representing only 5–10 % of added glycine-N. The results of the perfusion without added substrate revealed no significant changes during the initial period. The changes in the N content of the perfused organs were negligible. The NH_2-N content in the final perfusion fluid corresponded approximately to the amount of glycine-N added at the beginning of the experiment (34·3 % added as glycine-N and 36·3 % found as NH_2-N at the end of the experimental period). The method of NH_2-N determination was checked by adding a known amount of glycine to a quantity of perfusion fluid, the NH_2-N of which had been estimated while in circulation. The estimated NH_2-N values were found to agree satisfactorily with the calculated amounts, the average experimental error being 6 %. By the isolation of glycine from the perfusion fluid it was ascertained that the NH_2-N found at the end of the experiment was in fact glycine-N. This was done by adding to 250 ml. of the fluid, 100 ml. 2 *N* HCl and 100 ml. 5 % $HgCl_2$, filtering the precipitate, removing the Hg by H_2S, neutralizing and finally concentrating *in vacuo* to *c.* 20 ml. A small precipitate filtered off was disregarded. To the clear filtrate an ethereal solution of 3 g. β-naphthalenesulphochloride was added and the mixture was shaken for 6 hr. during which 24 ml. of *N* NaOH were gradually added. The mixture was again filtered and acidified with 2 *N* HCl, the precipitate filtered and recrystallized from water. The yield varied in the different experiments from 38 to 62 % of the amount expected in accordance with the NH_2-N determinations. The substance was β-naphthalenesulphoglycine. (Found: C, 54·08; H, 4·39; N, 5·53; S, 11·9. Calc. for $C_{12}H_{11}O_4NS$: C, 54·3; H, 4·16; N, 5·28; S, 12·07.) No other NH_2 compound was found to be present in the perfusion fluid.

Further experiments (not recorded) were made to investigate the effect of fixatives on the deamination of glycine. In liver slices no NH_3 formation or disappearance of NH_2-N occurred, however, with glycine in presence of fixatives. Ornithine was added as fixative for NH_3 and $NaHSO_3$ for glyoxylic acid, possibly formed. The presence of glucose also had no effect when added with glycine to kidney slices.

Further, the possibility of a collaboration of more than one organ in the metabolism of glycine was considered; since as mentioned earlier glycine administered *per os* appears to be readily attacked. The example of the formation of uric acid by collaboration of liver and kidney, as shown by Schuler & Reindel [1933], inspired these investigations. Mixtures of kidney and liver tissues and of kidney and spleen tissues were chosen.

In the experiments in which glycine was added to such mixtures (Table VIII) it will be seen, in the case of kidney slices plus spleen slices, that while no NH_3 or urea was formed, NH_2-N disappeared in some cases to an extent of approximately 70–95 % of the NH_2-N added. No disappearance of NH_2-N however was observed in the case of the mixture of kidney and liver slices, irrespective of the addition of substrates such as alloxan or ketoglutaric acid. (Alloxan is known to catalyse the oxidation of amino-acids *in vitro* [Strecker, 1862] and α-ketoglutaric acid has been reported to serve as an "acceptor" for amino groups [Braunstein & Kritzman, 1937, 1].) Kidney slices and kidney extract produced equal effects when mixed with the spleen slices.

Estimations of total N were carried out on the deproteinized filtrates of the experimental fluid. An example is given in Table VIII. No change of total N was observed, hence the NH_2-N disappearing was not used for synthesis of proteins.

Table VIII. *N changes and O_2 consumption in mixtures of tissue slices in presence of glycine*

Duration of exp.: 2 hr. Amount of glycine-N added: 1·12 mg. except in exp. † where 0·96 mg. was used. Rats were used for all tissue slices. Concentration of kidney extract: 50 mg. dried powder from pig kidney.

Mixture of tissue used	Extra NH_2-N with added glycine mg.	mg. NH_2-N				Extra O_2 consumption with added alanine, μl. after 60 min.	% of glycine-N disappeared
		Without glycine	With glycine	Difference	Disappeared		
Kidney slices† +Liver slices	—	0·20	1·07	0·87	0·09	—	9
Kidney slices† +Liver slices +Alloxan $M/100$	−0·02	0·17	1·14	0·97	0	− 2	0
Kidney slices +Liver slices +Ketoglutaric-acid $M/40$	−0·01	0·17	1·22	1·05	0·07	−38	7
Kidney slices +Spleen slices	0·04	0·38	0·63	0·25	0·87	49	78
Kidney slices +Spleen slices	0·02	—	—	—	—	—	—
Kidney slices +Spleen slices	0·03	0·34	0·61	0·27	0·84	—	75
Kidney extract +Spleen slices	0·10	—	—	—	—	−12	—
Kidney extract +Spleen slices*	0	0·73	0·80	0·07	1·05	29	94
Kidney extract +Spleen slices	0·01	0·48	0·81	0·33	0·79	—	70

	mg. Total N			
	Without glycine	With glycine	Difference	Disapp.
Estimation of total N in exp. *	0·06	1·20	1·14	0

The disappearance of NH_2-N in presence of mixed kidney and spleen tissue, not occurring in presence of either of the tissues alone, may be interpreted either as an enzymic effect or an effect of non-enzymic substances present in the tissue mixture. In order to answer this question, further experiments with *Kochsaft* of various tissues were carried out. The results are shown in Table IX. Kidney *Kochsaft* added to kidney slices caused a disappearance of approximately 25 % of the NH_2-N. Spleen *Kochsaft* alone had about the same effect, which was hardly increased by addition of kidney *Kochsaft*, but which appeared to be a little intensified in presence of kidney slices. Heart *Kochsaft* caused no effect. A comparison of the results of Table VIII with those of Table IX shows that the effect of *Kochsaft* alone or of spleen *Kochsaft* plus kidney slices on the disappearance of NH_2-N was considerably smaller than that of kidney slices plus spleen slices. O_2 consumption was slightly inhibited with added glycine, as in previous experiments, and extra NH_3-N formed in presence of glycine was insignificant.

The partial disappearance of NH_2-N under the experimental conditions described, which was not accompanied by a corresponding NH_3 or urea formation led to an investigation of other products of decomposition of glycine. The possibility of the formation of amides and creatine was considered next.

Table IX. *Experiments with kidney slices and kidney* Kochsaft *in presence of glycine*

Tissue	Substrate other than glycine	mg. NH_2-N: Without glycine	With glycine	Difference	Disapp. of NH_2-N	Disapp. of NH_2-N in % of added NH_2-N	Glycine-N added mg.	Other observations
Kidney slices	Kidney *Kochsaft*	0·22	1·10	0·88	0·27	**23**	1·15	Extra O_2 with glycine = −46
Kidney slices	Kidney *Kochsaft*	0·19	1·02	0·83	0·32	**28**	1·15	Extra O_2 with glycine = −13
Kidney slices	Kidney *Kochsaft*	0·14	0·96	0·82	0·33	**29**	1·15	Extra NH_3 0·15 mg.
Kidney slices	Kidney *Kochsaft**	0·32	1·18	0·86	0·29	**25**	1·15	Extra NH_3 0·09 mg.
Kidney slices	Heart *Kochsaft*	0·16	1·27	1·11	0	**0·06**	1·17	—
Kidney slices	—	0·01	1·23	1·22	0	**0**	1·12	—
Kidney slices	Kidney *Kochsaft*	0·16	1·00	0·84	0·28	**25**	1·12	Creatine test (Tintometer reading) **60** % increase with glycine
Kidney slices	Spleen *Kochsaft*	0·27	1·04	0·77	0·35	**31**	1·12	—
Kidney *Kochsaft*	Spleen *Kochsaft*	0·28	1·14	0·86	0·26	**23**	1·12	—
—	Spleen *Kochsaft*	0·14	1·02	0·88	0·24	**21**	1·12	—

Note. The *Kochsaft* was prepared by boiling 1 g. rat kidney for 7 min. on the water bath and centrifuging. 0·3 ml. of the supernatant fluid was used for all exp. except for exp. * where 0·6 ml. was used.

The formation of amide-N from glutamic acid has been recorded by Krebs [1935, 2]. No appreciable amount of amide-N however was found such as to justify the suggestion that the glycine-N disappeared had been retained as amide-N.

The formation of creatine from glycine has often been claimed, since numerous investigators have proved that patients suffering from the muscular dystrophies excrete less creatine, and since Brand *et al.* [1929; 1931] had found in these cases that following the injection of glycine creatinuria was increased. The same effect was observed after the administration of glycocyamine [Bodansky, 1936]. This has been isolated from the urine of dystrophic patients following administration of glycine [Weber, 1935]. It is generally assumed that glycocyamine acts as a precursor to creatine, the former being methylated by glycine to form the latter. Evidence for the conversion of glycocyamine into creatine in the body has been given by early workers [Jaffe, 1906; Thompson, 1917]. The methylation was assumed to take place in muscles and in kidney [Bodansky, 1936], although Shibuya [1937] failed to show such an effect in *in vitro* experiments with liver slices, and obtained doubtful results when perfusing liver of rabbits. Finally Davenport *et al.* [1938] supplied further evidence for this assumption.

Glycocyamine was added to mixtures of kidney and liver slices, in order to see whether it was able to cause NH_2-N disappearance as did *Kochsaft*. It will be seen from Table X that in a mixture of kidney and liver slices with added glycine, 46 % of the NH_2-N disappeared in presence of glycocyamine, whereas no appreciable disappearance of NH_2-N occurred when glycine alone was added. It will also be observed that the individual tissues, i.e. liver alone and kidney alone, had no appreciable deaminating effect in presence of glycocyamine. Thus glycocyamine showed an effect similar to that of kidney or spleen *Kochsaft*. There is of course no evidence that glycocyamine is one of the active constituents of spleen *Kochsaft* or kidney *Kochsaft*, although glycocyamine has been isolated from the urine. The results of the following experiments on creatine formation, however, offer further support for the action of glycocyamine.

In one of the experiments of Table IX creatine was determined approximately, and it was found to be present in a larger amount in presence of glycine than in its absence. More experiments of this type were made using chopped rat

Table X. *Disappearance of glycine-N in tissue slices in presence of glycocyamine*

Amount of glycine added: 1·12 mg. glycine-N, final concentration 0·2%. Amount of glycocyamine added: 1·5 mg., final concentration 0·05%.

Tissue	Substrate added other than glycine	mg. NH_2-N: Without glycine	With glycine	Difference	Disappearance of NH_2-N	Disappearance of NH_2-N as % of added NH_2-N
Kidney	Glycocyamine	0·1	1·12	1·02	0·1	9
Liver	Glycocyamine	0·1	1·03	0·93	0·19	17
Kidney + liver	Glycocyamine	0·2	0·81	0·61	0·51	46
Kidney + liver	—	0·2	1·07	0·87	0·09*	9
Kidney + spleen	Glycocyamine	0·35	1·12	0·77	0·35	31

* The amount of glycine-N added in this exp. was 0·96 mg.

Table XI. *Chopped heart of rat in presence of glycine and glycocyamine*

Concentration of glycocyamine: 0·1%. Amount of glycine added: 1·12 mg. glycine-N

Substrate added	NH_3-N mg.	Amide-N mg.	NH_2-N mg.	NH_2-N disapp. mg.	Creatine μg.	O_2 consumption
None	0·08	—	—	—	800	—
Glycine	0·26	—	—	—	740	154
Glycocyamine	0·19	—	—	—	770	135
Glycine + glycocyamine	0·22	—	—	—	1000	161
None	0·08	0·02	0·08	—	570	93
Glycine	0·15	0·03	0·09	0·11	570	84
Glycine + glycocyamine	0·19	0·08	0·92	0·28	710	98

heart and adding glycocyamine and glycine. The more reliable results of this series, as the technique for creatine estimation had to be improved in the course of this experimental work, are shown in Table XI. It will be seen that no extra creatine is formed with either glycine alone or glycocyamine alone, but small creatine rises were observed where glycine and glycocyamine had simultaneously been added. Furthermore, the disappearance of NH_2-N was observed to be greatest when both substrates had been added. It will be seen that in these experiments small but definite NH_3 formation has taken place. Again a small increase in O_2 consumption was observed with added glycine + glycocyamine.

These results may point to a possible *in vitro* methylation of glycocyamine by means of glycine, but the difficulties of estimating accurately small amounts of creatine prevent any definite conclusion in this respect.

Amongst substances possibly responsible for the disappearance of NH_2-N coenzymes had to be considered. The coenzyme responsible for the deamination of the unnatural amino-acids recently discovered by Warburg & Christian [1938] and Straub [1938] exists in sufficient quantities in most of the tissues and tissue extracts used. Hence an effect by adding it could not be expected.

Table XII shows experiments with cozymase and coenzyme II added to glycine under anaerobic conditions. Coenzyme II showed no effect. Cozymase usually caused a disappearance of NH_2-N of 40–65%, but sometimes much less.

Further experiments were made with pyruvic acid. Table XIII shows the results in kidney extract. They seem to be much more consistent than the experiments in Table XII and reveal that in presence of pyruvate the further

Table XII. *Effects of cozymase and coenzyme II on the disappearance of glycine-N in tissue slices under anaerobic conditions*

Duration of exp.: 2 hr. Amount of glycine added: 1·12 mg. glycine-N.

		With added glycine	
Tissue	Addition	NH_2-N disappearance mg.	NH_2-N disappearance as % of added NH_2-N
Kidney slices	Cozymase	0·74	66
Kidney slices	None	0	0
Kidney slices	Cozymase	0·48	43
Kidney slices	Cozymase	0·71	62·5
Kidney slices	Cozymase	0·12	10
Liver slices	Cozymase	0	0
Kidney slices	Cozymase	0·08	7
Kidney slices	Coenzyme II	0·14	12·5
Kidney slices	Coenzyme II	0·01	0
Liver slices	None	0	0

Table XIII. *Effect of pyruvate on the disappearance of glycine-N in kidney extract under anaerobic conditions*

Duration of exp.: 2 hr. Amount of glycine added: 1·12 mg. glycine-N (1·02 mg. in exp. *). Concentration of pyruvate: 1%.

		With added glycine	
Tissue extract	Substrates added	NH_2-N disappearance mg.	NH_2-N disappearance as % of added NH_2-N
Kidney extract	Cozymase + pyruvate	0·47	42
	Cozymase + pyruvate	0·39	35
	Pyruvate	0·41	36·5
	Pyruvate	0·40	35
	Pyruvate	0·61	40·5*
Boiled kidney extract	Pyruvate	0·21	21*
	Pyruvate	0·24	21
Water control (no tissue extract)	Pyruvate	0 (aerobic)	0 (aerobic)
	Pyruvate	0·20	18
	Pyruvate	0·16	15·5*

addition of cozymase is unnecessary for the disappearance of NH_2-N. 35–42% glycine-N disappeared in presence of pyruvate and in presence or absence of cozymase. Boiled extract had a small effect compared with unboiled extract. Under aerobic conditions no disappearance of NH_2-N occurred when tissue extract was replaced by water, whereas, under anaerobic conditions, a small disappearance was observed. This suggested some catalytic effect of the tissue.

In some examples of large disappearance of NH_2-N, estimations of NH_3 and urea-N, amide-N, total N and purine-N were performed simultaneously. There was no appreciable change observed in any of the N fractions tested, except in NH_2-N (Table XIV). This meant that the disappearance of NH_2-N could not be accounted for by the formation of any of the above N compounds, and suggested that the glycine had not lost its N but had condensed with another substrate, using in this way its NH_2 group to form a nitrogenous condensation product.

In estimating the simultaneous disappearance of pyruvic acid with the disappearance of NH_2-N the decomposition of a possible condensation product by the

Table XIV. *Simultaneous determination of nitrogenous substances in exp. with added glycine and pyruvate under anaerobic conditions*

Biological material: kidney extract.

	mg. N with added glycine		
	Initial	Final	Change
NH_3 + urea-N	0·08	0·08	0
	0·08	0·11	0·03
NH_2-N	1·29	0·54	−0·75
	1·38	0·99	−0·39
Amide-N*	0·10	0·10	0
Purine-N	0·38	0·38	0
Total N	1·96	1·90	−0·06
	1·77	1·68	−0·09
Pyruvate*	2·90	1·95	−0·95
	3·84	2·77	−1·07

* In these exps. kidney slices were used.

addition of $NaHSO_3$, used for the estimation of pyruvic acid, presented a difficulty. When the estimation was performed quickly, however, a disappearance of pyruvic acid was observed with added glycine (Table XIV).

It will be recalled that in almost every experiment recorded the O_2 consumption of tissue slices and extracts was slightly inhibited in presence of glycine. As a result of the experiments described with added *Kochsaft*, glycocyamine, pyruvate etc. a theory of condensation of certain factors with glycine has been advanced. Now, most of the substances mentioned are known to increase O_2 consumption when added to tissue in absence of glycine. It seems feasible, therefore, that if a condensation of glycine with such substances occurs, they may be prevented from increasing the O_2 consumption. From Fig. 4 it will be seen

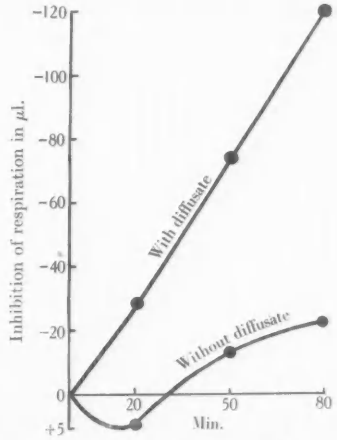

Fig. 4. The effect of tissue diffusate on the inhibition of tissue respiration by glycine.

that the inhibition by glycine is intensified in presence of added tissue diffusate. The latter was prepared by shaking 100 mg. kidney slices in 3 ml. phosphate buffer solution for 2 hr. 1 ml. of this solution was added to another portion of kidney slices suspended in 2 ml. buffer solution.

Discussion

The possibility of amino-transfer

When it was demonstrated in the first part of this work that glycine in absence of any further substrate is not metabolized in tissue extracts, tissue slices or in perfusion experiments with liver and kidney, because no NH_3 or urea was formed and no NH_2-N disappeared, it must be remembered that similar observations have been made before with other amino-acids, which led to different conclusions. The first observations of Needham [1930], who found an increase in succinic, fumaric and malic acids, but no change in either NH_2-N, NH_3-N, or amide-N, when glutamic and aspartic acids were incubated with minced muscle, were followed more recently by the experiments of Braunstein & Kritzmann [1937, 1, 2] and on the amino-transfer of amino-acids [Knoop & Oesterlin, 1925]. The former workers observed the formation of glutamic acid from glycine in presence of α-ketoglutaric acid. No change in the NH_2-N content can be expected under these conditions, i.e. the observations made by these workers are similar to those in the "negative" cases of this experimental work. But it may be argued that had amino-transfer taken place in those cases the result would have been the formation of another amino-acid from a suitable keto compound present, and deamination of this newly formed amino-acid would probably have been the necessary consequence. Thus, kidney tissue containing strongly deaminating enzymes would have given rise to NH_3. This was generally not observed in these "negative" cases, although an NH_3 formation of 8–10% of the NH_2-N added, and a corresponding NH_2-N disappearance of about the same order, occurred in isolated cases. If these small changes in NH_2-N can be considered as significant, the possibility remains that apart from the mechanism suggested by the later experiments, such a deamination either of glycine direct or of an amino-acid formed by the amino-transfer of glycine may take place to a small extent, explaining the findings of other workers who reported NH_3 formation from glycine of about the same order. These small amounts of NH_3 have in accordance with these other workers been observed under aerobic conditions only.

The astonishing stability of glycine towards organs, whether extracted, sliced or perfused, gives the impression that a more complicated mechanism than generally attributed to the process of deamination of amino-acids may exist, involving simultaneous contributions by various factors. It seems difficult, therefore, to localize the metabolism of glycine in a single organ without the concept of a contribution by compounds originating in other organs and introduced by the blood stream. This is probably why it was possible to show deamination of glycine administered *per os* or by injection, thus involving the whole metabolic system of the body. Attempts to show *in vitro* effects on glycine incubated with single organs were either frustrated or led to inconsistent results possibly depending on the accidental presence of preformed compounds necessary for the metabolic reaction. The latter could only consistently be observed when metabolites or enzymes of one organ were introduced into the enzymic system of another, thus setting up the necessary system for a metabolic attack on glycine.

The enzymic nature of the reaction

The experiments described may give some indication as to whether or not enzymes participate in the reactions observed. For the sake of the following

considerations, it may be permissible to divide the effects of the different biological materials on the disappearance of glycine-N into three groups:

group− causing a disappearance of 0–10 %,
group+ causing a disappearance of 10–30 %,
group++ causing a disappearance of 30–80 %.

The following table summarizes the results of the experiments with different tissues:

1. Kidney slices and kidney extract	−
2. Liver slices and other slices alone	−
3. Kidney+liver slices	−
4. Spleen *Kochsaft* alone	+
5. Kidney slices+kidney *Kochsaft*	+
6. Kidney *Kochsaft*+spleen *Kochsaft*	+
7. Kidney slices or kidney extract+spleen slices	++
8. Kidney slices+spleen *Kochsaft*	+

Thus under the experimental conditions described neither kidney nor any other organ alone, nor kidney+liver, is capable without further addition of compounds to cause disappearance of glycine-N (see 1, 2 and 3). Kidney+spleen slices causes a strong effect (see 7). The reaction between glycine and compounds present in spleen *Kochsaft* can take place to a small extent in absence of enzymes (see 4 and 6). The reaction responsible for the disappearance of glycine-N is catalyzed by enzymes present in kidney slices+spleen slices (compare 7 with 4, 5, 6 and 8).

Since in the experiments described the only metabolic change of glycine observed consisted in a disappearance of NH_2-N, a condensation of glycine with a non-enzymic component of the tissue was suggested. Two possible enzymic actions of the tissue may be therefore considered: either a catalytic effect on the mechanism of condensation, or an enzymic action, perhaps involving cozymase, which may lead to the production of tissue components with which the glycine condenses.

Results of the anaerobic experiments, with cozymase (Table XII) again indicate enzymic action of the tissue, since no effect was observed with kidney slices alone, whereas a definite disappearance of NH_2-N occurred in most experiments with kidney slices and added cozymase. Cozymase may therefore react either with the enzyme which catalyses the condensation, or cozymase may be concerned with the enzymic formation of suitable substrates for the condensation. The first possibility can be excluded by the results of the experiments with pyruvate (Table XIII), as cozymase+pyruvic acid and pyruvic acid alone exert identical effects. For the second question the nature of the non-enzymic components of the condensation has to be taken into consideration.

The nature of the non-enzymic components of the glycine condensation

(*a*) *Keto compounds.* These compounds play an essential part in the metabolism of kidney and liver, and condensation of amino-acids with aldehydes and keto bodies is well known to take place purely chemically [Schiff, 1901; Sørensen, 1908]. More recently a similar simple condensation has been described by Herbst [1936] between amino-acids and pyruvic acid. Akasi [1937] reports the isolation of octopine, i.e. a reduced Schiff's base, from muscle of Octopoda: this has recently been synthesized by Knoop & Martius [1938] by condensation of pyruvic acid with arginine.

Pyruvate was shown in fact to cause disappearance of NH_2-N, and here again, the failure to detect a production of NH_3 and other nitrogenous products of decomposition strongly suggested the formation of a product of condensation of glycine with pyruvate. This suggestion was supported by a disappearance of pyruvic acid simultaneously with the NH_2-N as observed in the cases recorded. The lability of such a condensation product probably prevented the establishment of a clear stoichiometric comparison between the disappearance of NH_2-N and pyruvate. Finally, the fact that the presence of cozymase was required for the reaction with kidney slices alone, but not when pyruvate was added, suggested a link with dehydrogenase systems known to be present in kidney, by means of which keto bodies may be formed. Thus pyruvic acid possibly produced by the lactic-pyruvate dehydrogenase system represents an example of such a reaction. Other keto-compound systems, such as hydroxybutyric-oxaloacetic or more likely the malic-oxaloacetic dehydrogenase, may be taken into consideration, the last being reported to be present in high concentration in kidney [Green, 1936]. Adler *et al.* [1938] consider that the fixation of NH_3 by keto-acids in animal cells takes place with the help of aspartic dehydrogenase, the activity of which is dependent on cozymase. Although no evidence of a direct link between such dehydrogenase systems and the disappearance of NH_2-N has been produced in this work, it may be pictured that the equilibrium of such a system may be shifted towards the formation of keto compounds in presence of glycine as a fixative for such keto compounds. This problem will be a matter of further investigation.

(*b*) *Glycocyamine.* The underlying idea of these experiments was the general methylating capability of glycine as shown by a number of workers [Hoppe-Seyler, 1930; Challenger, 1936] and the special methylating action of glycine on glycocyamine to form creatine (*v. supra*). The mechanism of the methylation of glycocyamine by glycine may be pictured to take place as follows:

$$CH_2NH_2COOH + O \rightarrow CHO.COOH + NH_3 \qquad \text{.....(I)}$$

$$CHO.COOH + \underset{\underset{NH_2}{|}}{\overset{\overset{NH}{\|}}{C}}.NH.CH_2COOH \rightarrow \underset{\underset{NH_2}{|}}{\overset{\overset{NH}{\|}}{C}}.N.CHOH.COOH \qquad \text{.....(II)}$$

$$\xrightarrow[\text{Decarboxylation}]{\text{Reduction}} \underset{\underset{NH_2}{|}}{\overset{\overset{NH}{\|}}{C}}.N(CH_3).CH_2.COOH + CO_2 + H_2O$$

The condensation of the deaminated glycine with glycocyamine is presented above in a scheme slightly different from that set up by other workers. Glyoxylic acid was considered to be an easier condensing material than glycollic acid which has been postulated by other workers, to act as the methylating agent following the deamination of glycine. The former view is supported by findings of Milhorat & Toscani [1936] who obtained a greater rise in creatine, when glycine was administered to patients with muscular dystrophy than when glycollic acid was used. The scheme postulates as the first step a deamination of glycine. According to Table XI a small NH_3 production was observed when glycine or glycocyamine was added to heart tissue. Thus this was the only tissue tested in which glycine was shown to produce NH_3 on incubation. Furthermore, the O_2 consumption was definitely increased with glycocyamine + glycine whereas

it was decreased with either glycine or glycocyamine alone. Although the changes mentioned are small, they may support the evidence for the formation of creatine *in vitro*.

General metabolic action of glycine

It was the object of these experiments to demonstrate the principle of condensation of glycine in place of its simple deamination. Many more possible condensations are conceivable, a number of which are known to take place, such as the formation of hippuric acid by condensation with benzoic acid, the condensation to carbamidoacetic acid [Hill, 1932], by condensation with alloxan, and possibly others. Owing to its instability the product of condensation with keto bodies cannot be regarded as an end-product in the metabolic path. The compound may either undergo tautomerization as suggested by Braunstein & Kritzmann [1937, 1, 2] and Knoop & Martius [1938] followed by splitting with the formation of a new amino-acid, thus completing amino-transfer. Or it may be reduced similarly to octopine and thus be stabilized. In this way glycine may act as fixative for keto compounds producing an equilibrium effect in systems responsible for the formation of such compounds.

The latter phenomenon may lead to a basis of the well-known "specific dynamic effect" of glycine by picturing its role as an acceptor of aldehydes and keto products thus catalysing the reaction within the enzymic systems concerned. By its condensing capacity it may act as a general reagent for the removal of compounds the metabolism of which would otherwise come to a standstill.

SUMMARY

1. Glycine, unlike alanine and other amino-acids, is little or not at all deaminated when incubated with slices of kidney, liver, spleen, diaphragm and brain of rat, or with extracts of kidney, testis and other organs. The NH_3 and urea formation and the disappearance of NH_2-N does not exceed 8% of that corresponding to the amount of glycine added and is not increased by fixatives such as $NaHSO_3$ and ornithine. 65–100% of added *dl*-alanine (calculated on the basis of the unnatural isomeride) was deaminated under the same conditions. The deamination of alanine was not increased by the addition of glycine.

No NH_3 and urea formation was observed during the perfusion of liver and kidney of the cat with glycine. At the end of the experiment all the glycine was recovered as NH_2-N. Glycine was isolated from the perfusion fluid as the β-naphthalenesulpho-derivative.

2. When glycine was incubated with a mixture of kidney and spleen slices or kidney extract + spleen slices, a disappearance of 70–80% of the NH_2-N was observed, without a corresponding formation of NH_3 or urea. The total N content of the deproteinized fluid was unchanged before and after the experiment. On the other hand a mixture of kidney and liver slices with or without added activators, for example alloxan, caused no such effect. From experiments using kidney slices with added *Kochsaft* of kidney or spleen a condensation of glycine with heat stable compounds under the influence of kidney and spleen enzymes is suggested.

3. O_2 consumption of tissue slices and extracts is slightly inhibited by glycine. This phenomenon is believed to be caused by its condensation with oxidizable compounds, thus protecting them from oxidation. Under the same

experimental conditions the oxidation of *dl*-alanine corresponds to the theoretical amount required for oxidative deamination of the unnatural isomeride.

4. Glycocyamine was shown to cause a disappearance of NH_2-N similar to that produced by kidney and spleen *Kochsaft* when added to glycine in presence of mixtures of tissue slices.

5. A small creatine and NH_3 formation was demonstrated *in vitro* from glycine when incubated with heart tissue in presence of glycocyamine. A scheme is suggested for the condensation of glycine with glycocyamine to form creatine.

6. 40–60 % of the NH_2-N disappeared when glycine was incubated with kidney slices or kidney extract in presence of cozymase or pyruvate under anaerobic conditions. The effect was more consistent with pyruvate, which partly disappeared simultaneously, and was not increased with further addition of cozymase. The role of the latter is believed to be concerned with the formation of keto compounds. No change was observed in these experiments in the content of NH_3-, amide-, purine- and total N before and after the experiment. Pyruvate, when incubated with glycine in presence of boiled kidney extract under anaerobic conditions, and even in absence of tissue, induced a small disappearance of NH_2-N, but none in the absence of tissue under aerobic conditions.

7. The possible metabolic pathways of glycine are discussed with special reference to amino-transfer. A general tendency of glycine to condense with glycocyamine, keto-bodies and other compounds is suggested. The influence of cozymase, possibly introduced into the system by *Kochsaft*, may point to a link between dehydrogenase systems concerned with the formation of keto-compounds (pyruvic acid, oxaloacetic acid etc.), and the above-described condensation of glycine with such compounds. Glycine may thus shift the equilibrium in such systems in the direction of increased oxidative production of keto-acids, a possibility that would explain its well-known specific dynamic effect.

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XIII. THE REPRESENTATIVENESS OF EXTRACTED SAMPLES AND THE EFFICIENCY OF EXTRACTION OF PROTEIN FROM THE FRESH LEAVES OF PLANTS; AND SOME PARTIAL ANALYSES OF THE WHOLE PROTEINS OF LEAVES

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Although from the time of Rouelle [1773] a method had existed for the extraction of impure protein samples from the fresh leaves of plants, very little extraction work was done until Osborne & Wakeman [1920] macerated spinach leaves and obtained protein from the juice. Since 1920 most of the work has been done by Chibnall and co-workers, with progressive improvements in technique: Chibnall & Schryver [1921]; Chibnall [1922; 1923; 1924]; Chibnall & Grover [1926]; Miller & Chibnall [1932]; and Chibnall *et al.* [1933].

By repeated maceration of spinach leaves in the presence of water Osborne & Wakeman [1920] extracted all but 16% of the nitrogen. They centrifuged the green juice to remove cell debris and flocculated two fractions of protein by adding successive portions of alcohol, the fractions, after purification by extraction with solvents, containing 12·9% N and 14·9% N respectively. Subsequently, Osborne *et al.* [1921] fractionated the nitrogenous constituents of lucerne leaves by macerating and extracting with various solvents, and obtained fairly pure specimens of protein by filtering the extracts through paper pulp before flocculating. Of the 56% of the leaf N which was not obtained in aqueous solution very little could be extracted by 93% ethyl alcohol or 0·075 *N* NaOH solution, but nearly 40% was extracted by 0·075 *N* NaOH in 60% alcohol. They noticed that the chloroplast material in the aqueous extract was removed by filtration. Considerable protein was flocculated from the filtrates of the aqueous and alkaline water-alcohol extracts.

The attention of Chibnall and co-workers has frequently been directed towards the preparation of protein samples in as high a state of purity as possible. Chibnall [1923] observed that the readily diffusible constituents of the leaf cells could be removed very well by plasmolysing with ether and other anaesthetics and pressing the leaves. Subsequently ether-water and "used" ether-water (i.e. ether-water already used once for the purpose) were substituted for ether, because they permitted better extraction of the protein when the leaves were macerated later in the presence of water. The juices, thus freed in advance from most of the diffusible solutes, were filtered through paper-pulp and the proteins were flocculated, coagulated and purified by extraction with various organic solvents. The yields have varied greatly (from 1 or 2 to 40% of the leaf protein), but some fairly "pure" preparations have been made. Using ether as plasmolysing agent

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preparations have been obtained from spinach and broad-bean leaves containing 15·95 % N and 1·7 % ash, and 16·05 % N and 4·3 % ash, respectively. These are the highest N contents of any preparations yet obtained from leaves.

With the object of improving the purity of preparations Osborne & Wakeman [1920], Chibnall & Schryver [1921] and Davies [1926] dissolved them in alkali and reflocculated with acid, and were usually successful in increasing the percentages of N. Foreman [1938] obtained small yields of relatively pure preparations by special techniques which did not involve solution in alkali.

There have been two aims in most of this protein extraction work: (1) to obtain large yields, in the belief that high-yield preparations are likely to be more representative of the whole protein in the leaves, and (2) to obtain "pure" preparations because of the difficulties and uncertainties which may attend the analyses of impure preparations. But unfortunately the two aims have usually proved to be mutually exclusive, and it has long been desirable to have some means of checking the representativeness of extracted protein samples. The only accessible means of classifying them and comparing them with the whole leaf-protein of which they represent a part and with the residual leaf-protein (i.e. that not extracted), is through their compositions. An extracted protein preparation may contain some 15 % N whereas, after removal of non-protein N, the whole leaf and the residue may contain only 3 or 4 % N. The methods of analysis employed must therefore be free from interference by very large amounts of impurities, and there are not, as yet, many such methods. Miller [1935] estimated the lysine, arginine and histidine contents of whole and extracted leaf-proteins of cocksfoot grass, and believed that the differences encountered, though fairly large, were of uncertain significance. The object of the present work was to obtain information of the composition of whole leaf-proteins, and to test the representativeness of samples of protein extracted by methods already in use and by any others which suggested themselves.

All materials from which non-protein N has been removed, whether they represent the whole leaf, the extracted preparations or the leaf residues, will be referred to hereafter as "protein preparations" and will be differentiated where necessary by the self-explanatory prefixes "whole", "extracted" and "residual" and the corresponding abbreviations W, E and R.

Experiments and discussion

The preparations (general)

Details concerning the fresh leaf materials and the extraction of samples of protein are contained in Tables I and II. The contents of Table II are explained later in the text and in accompanying remarks. Table I shows in the order of the columns: the label of the protein preparation (exp. number, followed by abbreviation showing whether "whole", "extracted" or "residual" and sometimes other distinguishing signs): the leaf-material source and the date: the percentage dry weight of the leaf material: the percentage of total N (t), coagulable N (c) and protein N (p) in the dry leaf: abbreviations indicating the method of extracting the sample of protein (if an extracted preparation): the percentage of the leaf N (t, c and p) appearing in an extracted juice or leaf residue: the percentage of N in the dry protein preparation: and finally the percentage of ash in it after ignition in air at about 600°. Sometimes a few % more or less total N could be found in the juice plus residue than was calculated to have been present in the fresh leaf material used. As there was never any definite evidence of real loss of N

Table I

Prep.	Source and date		% dry wt.	% N in dry leaf (*t*, *c*, *p*)	Method of extraction	% extracted and residual N (*t*, *c*, *p*)	% N in prep.	% ash prep.
1W	*Dactylis glomerata* (cocksfoot grass)	29. ix. 36	12·5	6·81*t*, — 4·50*p*	—	— — —	7·53	3·2
1E	,,	,,			*w*, *c*, *w*, *p*, , *f*.	— — 39·4*p*	14·1	1·1
2W	,,	7. vi. 37	19·0	4·05*t*, — 3·36*p*	—	— — —	5·61	5·6
2E(*a*)	,,	,,			*w*, *c*, *w*, *p*, , *f*.	— — *ca*. 20*p*	13·05	1·2
2E(*b*)	,,	,,			*d*, *fs*, *w*, *p*, , *f*.	71·5*t*, — 61·1*p*	12·6	1·4
3W	*Lolium perenne* (perennial rye-grass)	9. vi. 37	15·5	4·19*t*, — 3·38*p*	—	— — —	4·93	23·2
3E(*a*)	,,	,,			*w*, *c*, *w*, *p*, , *f*.	— — *ca*. 20*p*	13·55	1·4
3E(*b*)	,,	,,			*d*, *fs*, *w*, *p*, , *f*.	— — 59·0*p*	12·35	6·4
4W	,,	29. xi. 37	17·8	4·20*t*, 3·14*c*, 3·00*p*	—	— — —	5·12	13·3
4E′	,,	,,			*d*, *c*, *p*H 7, *p*, , *c*.	45·1*t*, 29·4*c*, 27·8*p*	13·1	7·3
4E″	,,	,,			, , *p*H 10, *p*, , *c*.	4·1*t*, 3·2*c*, 3·1*p*	8·15	25·8
4R	,,	,,			—	50·8*t*, 63·7*c*, 62·7*p*	3·45	19·9
5W	*Beta cicla* var. (beet spinach)	3. xii. 37	10·3	4·17*t*, 3·17*c*, 3·02*p*	—	— — —	6·14	9·7
5E′	,,	,,			*d*, *c*, *p*H 7, *p*, , *c*.	46·9*t*, 30·7*c*, 29·4*p*	13·3	6·3
5E″	,,	,,			, , *p*H 10, *p*, , *c*.	4·6*t*, 3·1*c*, 2·9*p*	12·1	7·6
5R	,,	,,			—	48·5*t*, 60·4*c*, 58·9*p*	4·06	12·8
6W	*Festuca ovina* (sheep's fescue grass)	19. v. 38	22·7	3·14*t*, 2·52*c*, 2·41*p*	—	— — —	—	—
6	[See Table II]							
7W	*Lolium perenne* (perennial rye-grass)	8. vi. 38	13·0	4·43*t*, 3·60*c*, 3·40*p*	—	— — —	5·77	4·7
7E	,,	,,			*d*, *c*, *p*H 9, *sc*, , .	56·2*t*, — 46·5*p*	12·5	3·7
7R	,,	,,			—	43·8*t*, — 48·5*p*	3·54	5·6
7E1	,,	,,			*d*, *c*, *p*H 9, *sc*, *ae*, *c*.	50·7*t*, — 46·1*p*	13·2	3·0
7RE1	,,	,,			—	5·5*t*, — 4·6*p*	8·5	—
7E2	,,	,,			*d*, *c*, *p*H 9, *sc*, *ae*, *f*.	36·8*t*, — 30·2*p*	14·65	0·6
8W	*Dactylis glomerata* (cocksfoot grass)	4. x. 38	17·1	7·34*t*, 6·16*c*, 5·92*p*	—	— — —	8·91	3·9
8E1	,,	,,			*d*, *f*, *p*H 9, *sc*, , .	92·1*t*, 86·7*c*, 85·9*p*	13·25	3·1
8E2	,,	,,			*d*, *f*, *p*H 9, *sc*, *ae*, .	92·1*t*, 94·1*c*, 94·1*p*	13·4	3·8
8R	,,	,,			—	7·9*t*, 8·8*c*, 8·4*p*	1·82	7·8
8E3	,,	,,			*d*, *f*, *p*H 9, *sc*, , *f*.	69·1*t*, 60·3*c*, 59·7*p*	14·2	0·6
8E4	,,	,,			*d*, *f*, *p*H 9, *sc*, *ae*, *c*.	87·5*t*, 89·0*c*, 88·8*p*	14·55	2·2
8RE4	,,	,,			—	4·6*t*, 5·2*c*, 4·7*p*	6·16	—
8E5	,,	,,			*d*, *f*, *p*H 9, *sc*, *ae*, *c*.	65·6*t*, 62·5*c*, 60·7*p*	15·0	0·6

Table II

8 g. portions of fresh leaves of *Festuca ovina* (22·7% dry wt.; 3·14% total N, 2·52% coagulable N and 2·41% protein N in the dry leaves) were macerated in the end-runner mill for 5 min. with 50 ml. of (i) water; (ii) *p*H 7·2 sodium phosphate buffer containing 1·5 g. P per litre; (iii) and (iv) *p*H 9·2 sodium borate buffer containing 1·1 g. B per litre; and the mash was centrifuged for 2 min. at 100 × gravity. From moisture content of leaves and moisture loss during maceration (estimated by tests with standard acid), the final volume of liquid was calculated to be 55·0 ml. Each 5 ml. therefore should have contained 5·17 mg. total N, 4·15 mg. coagulable N and 3·98 mg. protein N if all the N had been completely and homogeneously dispersed into the liquid. Compare with data in column (2).

In (i), (ii) and (iii), the 5 ml. aliquots of juice (left at *p*H 6 if water had been used, otherwise adjusted with alkali to buffer *p*H value) were centrifuged for about 30 min. at 25,000 × gravity to separate the granule from the non-granule fractions, judged by transparency of, and absence of "greenness" in the liquid layer. See columns (3) and (4). The granules, when later extracted with a protein solvent, were stirred with about 5 ml. of the solvent and the emulsion was left for 10 min. before recentrifuging. See columns (5)–(16). Data in column (17) were obtained from mixed extracts of the granules as indicated. The solvents were variously: 5 ml. of the aqueous solvents, water, diluted *p*H 7·2 buffer, diluted *p*H 9·2 buffer, and 0·05 *N* NaOH; 4·5 ml. of aqueous solvent plus 0·5 ml. of ether; 2·5 ml. of aqueous solvent plus 2·5 ml. of alcohol; and 2·5 ml. of aqueous solvent plus 2·0 ml. of alcohol and 0·5 ml. of ether.

In (iv) the juice itself was treated with a mixture of 0·8 vol. of alcohol and 0·2 vol. of ether before centrifuging. The figures in parentheses above certain values indicate the percentages of N in the dried precipitates concerned. The granule N: non-granule N ratio in (i) is believed to have been too high from sedimentation of a little non-granule protein (denatured?) as a pale top layer.

Form of N (1)	Juice M mg. (2)	Non-gran. N mg. (3)	Gran. N mg. (4)	Action of solvents upon granules: Sol. N mg. (5)	Insol. N mg. (6)	Sol. N mg. (7)	Insol. N mg. (8)	Sol. N mg. (9)	Insol. N mg. (10)	Sol. N mg. (11)	Insol. N mg. (12)	Sol. N mg. (13)	Insol. N mg. (14)	Sol. N mg. (15)	Insol. N mg. (16)	Sum of 5, 7 and 9 (17)
	(i)	Water		Water		Water ether		Water alcohol								
Total	4·69	2·96	1·73	0·14	1·59	0·21	1·52	0·18	1·55	—	—	—	—	—	—	0·51
Coagulable	3·50	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
Protein	3·35	1·74	(11·5) 1·61	—	—	—	—	—	—	—	—	—	—	—	—	0·18
	(ii)	*p*H 7·2 buffer		*p*H 7·2 buffer		*p*H 7·2 buffer ether		*p*H 7·2 buffer alcohol								
Total	4·93	3·24	1·69	0·21	1·48	0·44	1·25	0·57	1·12	—	—	—	—	—	—	1·22
Coagulable	3·80	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
Protein	3·63	(11·7) 2·06	1·57	—	—	—	—	—	—	—	—	—	—	—	—	(12·0) 0·93
	(iii)	*p*H 9·2 buffer		*p*H 9·2 buffer		*p*H 9·2 buffer ether		*p*H 9·2 buffer alcohol		*p*H 9·2 buffer alcohol-ether		0·05 *N* NaOH		0·05 *N* NaOH alcohol		
Total	5·01	3·27	(7·7) 1·74	0·34	1·40	0·67	1·07	0·97	0·77	1·20	0·54	0·53	1·21	1·35	0·39	1·98
Coagulable	3·86	—	—	—	—	—	—	—	—	—	(5·7) 0·52	—	—	—	—	—
Protein	3·68	(12·2) 2·08	(11·3) 1·60	—	—	—	—	—	—	—	(8·5) 0·42	—	—	—	—	(13·0) 1·74
	(iv)	*p*H 9·2 buffer add alcohol ether														
Total	5·01	4·41	(4·6) 0·60	—	—	—	—	—	—	—	—	—	—	—	—	—
Coagulable	—	—	0·58	—	—	—	—	—	—	—	—	—	—	—	—	—
Protein	3·89	(14·5) 3·42	0·47	—	—	—	—	—	—	—	—	—	—	—	—	—

(as NH_3, for example), the discrepancies were attributed to sampling errors. Values shown in the table have been adjusted as for perfect recovery of total N.

Of the abbreviations concerning the method of extracting protein from the leaves, *d* signifies direct maceration with the protein solvent, or *w*, maceration after removal of readily diffusible leaf-cell constituents by plasmolysis with "used" ether-water and pressure in the Chibnall *et al.* [1933] process. Following these abbreviations, *c* means that the material was coarsely macerated in a mincing-machine, *f*, that it was finely macerated in an end-runner mill, or *fs*, that it was finely macerated with 20 % by weight of sand in the runner mill. Then *w* signifies that water, or "*p*H 7 (or 9 or 10)" that a buffer solution of the indicated *p*H value, was the protein solvent with which the leaves were macerated. Following these, *p* signifies that the mash was pressed in a cloth, or *sc*, that it was sieved and the liquid centrifuged for 2 min. in a field of 100× gravity, to separate the juice from most of the cell debris. Then *ae* indicates that a cold mixture of 0·8 vol. alcohol and 0·2 vol. ether was stirred into the juice with cooling and the mixture allowed to stand for 15–30 min. Finally, *f* means that the juice was filtered through a pad of paper-pulp, or *c*, that it was centrifuged for 30 min. in a field of 500 × gravity, before the protein was flocculated. Blanks in the sequence of abbreviations indicate omitted steps. The extractions were done at room temperature (10–25°).

In general, a higher percentage dry weight of leaf in any one species reflects greater age of leaf; and, ages being similar, a higher percentage of N in the dry leaf reflects better manuring. Inasmuch as it is believed that the leaf proteins of the species would all contain some 17·0 % N if they could be obtained pure, the actual values for the preparations afford some measure of purity, and, in conjunction with the percentages of ash, some measure of organic impurity. It is believed that nitrogenous impurities were absent from the protein preparations.

Methods of estimation

Nitrogen estimations were made by the Kjeldahl method. The terms "coagulable nitrogen" and "protein nitrogen" have been explained in earlier articles [Lugg, 1938, 2, 3] and apply in the present article to the original leaf materials, to the extracted juices containing protein and other nitrogenous substances, and to the leaf residues after extracting juices. Briefly, "protein N" means N not extracted by successive applications of boiling alcohol, dilute citric acid, alcohol and ether, to a material containing "coagulable N"; and (usually) "coagulable N" means N left in insoluble material after heating at about 90° in dilute acetic acid at about *p*H 4·5. In some instances, to be discussed, coagulation was performed by heating to about 70° in water-alcohol mixtures (which sometimes contained ether until it had been boiled off) at a *p*H corresponding with that of a mixture of aqueous acetate *p*H 4·5 buffer with 0·8 vol. alcohol, and therefore above 4·5 but on a different *p*H scale (see, e.g. Richardson [1934]). By coagulating in presence of 0·8 vol. alcohol it was normally found that the coagulable N and protein N were increased in the case of a juice, but in the case of a dried leaf material they were almost unaffected as also were the percentages of N and ash in the resulting whole protein preparation.

Amide, tyrosine and tryptophan, and cystine plus cysteine and methionine estimations were made with the protein preparations by the methods described in the articles [Lugg, 1938, 1, 2, 3], wherein it has been shown that the estimations of amide, tyrosine, tryptophan, cystine plus cysteine (by differential oxidation) and methionine (by differential oxidation) are not subject to fresh systematic errors when some carbohydrates are added to a protein. It was

assumed that they would be satisfactory in the presence of much cellulose and other cell-wall constituents; and, in fact, satisfactory tests of the procedures for estimating tyrosine and tryptophan were made with much cellulose present. It was found too that amide values obtained with the whole and residual protein preparations by varying the hydrolysis procedure differed from those obtained by the standard procedure in much the same way as did values with extracted protein preparations [Lugg, 1938, 3]. Estimations with whole and residual protein preparations were sometimes made on a larger scale than was customarily adopted with extracted protein preparations.

The random errors of estimation with whole and residual protein preparations are believed to have been not more than about 1·5 times as great as they were in the case of extracted protein preparations, and these last are believed not to have exceeded the figures indicated in the following general description of Table III,

Table III

Prep.	%N	% amide N	% tyr.-N.	% try.-N	% cyst.-N (d.o.)	% meth.-N (d.o.)	% cyst. + meth.-N (d.o.)	SO_4-S × 100/N (d.o.)	% cyst.-N (HI t)	% meth.-N (HI t)
1W	7·53	5·10	2·44	1·90	1·38	1·46	2·84	1·07	—	—
1E	14·1	5·26	2·34	1·82	1·68	1·49	3·17	0·40	1·50	1·30
2W	5·61	5·04	2·53	1·78	1·41	1·61	3·02	0·36	—	—
2E(*a*)	13·05	4·95	2·32	1·80	1·55	1·42	2·97	0·78	1·35	1·22
2E(*b*)	12·6	5·02	2·39	1·88	1·44	1·52	2·96	0·65	—	—
3W	4·93	5·26	2·48	1·82	1·41	1·48	2·89	0·39	—	—
3E(*a*)	13·55	4·73	2·34	1·73	1·69	1·58	3·27	0·66	1·54	1·50
3E(*b*)	12·35	5·15	2·44	2·09	1·57	1·53	3·10	0·25	—	—
4W	5·12	5·20	2·41	1·78	1·47	1·59	3·06	0·76	—	—
4E′	13·1	4·96	2·43	2·05	1·63	1·52	3·15	1·12	1·20	1·07
4E″	8·15	5·64	2·28	1·62	1·48	1·49	2·97	1·30	—	—
4R	3·45	5·15	2·44	1·54	1·51	1·58	3·09	1·02	—	—
5W	6·14	5·28	2·63	1·50	1·45	1·43	2·88	0·63	—	—
5E′	13·3	5·18	2·57	2·01	1·47	1·44	2·91	0·91	1·24	1·17
5E″	12·1	5·18	2·68	1·71	1·59	1·39	2·98	0·73	—	—
5R	4·06	4·97	2·79	1·35	1·53	1·42	2·95	0·94	—	—
7W	5·77	5·21	2·45	1·92	1·42	1·50	2·92	0·62	—	—
7E	12·5	5·27	2·42	1·97	1·40	1·60	3·00	0·43	1·21	1·41
7R	3·54	5·30	2·51	1·88	1·48	1·45	2·93	0·74	—	—
7E1	13·3	5·15	2·35	1·92	1·46	1·47	2·93	0·34	1·17	1·36
7E2	14·65	5·17	2·39	1·96	1·37	1·46	2·83	0·34	1·25	1·40

which contains the results of analyses. In the order of the columns are shown: the characteristic label of the preparation: the percentage of N in it (error 0·5 %, affecting all the other errors): the amide content (error 1·5 %): tyrosine content (error 2 %): tryptophan content (error 3 %): cystine plus cysteine content, by differential oxidation (error 4 %): methionine content, by differential oxidation (error 5 %): the sum of the last two values (error 2 %): sulphate-S as a percentage of the weight of the N in the preparation (error from 3 % for the higher to 20 % for the lower values): cystine plus cysteine content, by titration of HI digests (error 3 %): and finally the methionine content, by titration of HI digests (error 4 %). The values have been corrected for such losses as the pure, free amino-acids undergo in the analytical procedures involved. To facilitate comparison of the compositions by way of proving the non-identity or assessing the probability of identity of the protein moieties of preparations (whole, extracted and residual), the results have been reported on a nitrogen basis, namely, as percentages of the N in the preparations appearing as the particular chemical species (amide or amino-acid) estimated. Although they are believed not to have

contained nitrogenous impurity, the preparations may have contained N in groups (such as nucleic acid) conjugated with some of the protein. Values in the last three columns cannot be used in comparing compositions. Those in the last two are probably low, though the precise significance of the disparities between them and the values obtained by the highly unspecific differential oxidation procedure is uncertain, whilst those in the previous column (sulphate-S) are to be regarded as measures of impurity.

(1) *Extraction test with* Dactylis glomerata

A check was made of the representativeness of protein extracted from a batch of *Dactylis glomerata* leaves by the method of Chibnall *et al.* [1933]. Details concerning the fresh leaf material (which was young and taken from a well-manured plot), and the whole and extracted protein preparations, are contained in Table I under the designations 1W and 1E. The results of partial analyses of the preparations are shown in Table III under the same designations. The S distributions were significantly different, the cystine plus cysteine content of 1E being considerably greater than that of 1W.

(2, 3) *Extraction tests with* Dactylis glomerata *and* Lolium perenne

The object of making the preparations 2W, 2E (*a*) and 2E (*b*) from leaves of *Dactylis glomerata*, and 3W, 3E (*a*) and 3E (*b*) from leaves of *Lolium perenne*, was to test again the representativeness of protein extracted by the method used in experiment 1 (giving now 2E (*a*) and 3E (*a*)) and further, to test the efficacy of a direct fine-maceration method which yielded 2E (*b*) and 3E (*b*). The surfaces of the *Lolium perenne* leaves were contaminated with soil. Details concerning the leaf materials and the extraction work are recorded in Table I, and analyses of the preparations in Table III. 2E (*a*) was of distinctly lower tyrosine content than 2W. Of 3E (*a*) and 3E (*b*), the former contained larger amounts of the sulphur-containing amino-acids and less amide than 3W, whilst the latter differed from 3W most noticeably in possessing a higher tryptophan content. Clearly, no significant improvement in the representativeness of extracted protein was achieved by macerating the leaves very finely; and, despite extensive extraction, the leaf residues having been macerated repeatedly with more water, considerable amounts of the leaf proteins were missing from the extracted preparations.

(4, 5) *Extraction tests with* Lolium perenne *and* Beta cicla

The unsatisfactory recoveries of protein in the fine-maceration tests in Exps. 2 and 3 suggested that much protein was being retained by the filter pads, possibly in association with the plastids and nuclei. In Exps. 4 and 5 (Table I) with leaves of *Lolium perenne* and *Beta cicla* respectively, it was accordingly decided to centrifuge, instead of filter, the juices which had been pressed through a cloth. The juices were centrifuged for 30 min. in a field of 500 × gravity, and although considerable chloroplast and other material was thus removed with cell-wall debris, the juices were still opalescent and strongly green in colour. A direct, coarse-maceration procedure was used in both experiments, and the initial extracting solvent was a *p*H 7 phosphate buffer containing 1·5 g. P per litre, providing preparations 4E′ and 5E′ respectively. The residues were then extracted with a *p*H 10 borate buffer containing 1·1 g. B per litre, but without further maceration, and yielded 4E″ and 5E″ respectively. It was hoped that

such nuclei, chloroplasts etc. as had been liberated from the cells but remained entangled with the leaf residues after the first extraction, would yield at least some of their protein to a *p*H 10 buffer. The high ash contents of the whole (4W and 5W), the extracted (4E′ and 4E″, and 5E′ and 5E″) and the residual (4R and 5R) protein preparations, are attributable to the fact that the leaf surfaces were contaminated with loamy soil.

The partial analyses of the preparations are recorded in Table III. They show that the proteins extracted at *p*H 10 differed considerably from those extracted at *p*H 7, and that the total extracted proteins were far from being representative of the whole proteins of the leaves. In neither case was the extracted plus residual protein N as great as the whole protein N. The losses were at first attributed to proteolytic activity in the extracts, but it was found later that the amounts of protein left in aqueous solution at *p*H 4·5, even after heating to 90°, are usually by no means negligible.

The one satisfactory aspect of these two experiments, 4 and 5, was that after making rough corrections for protein losses, all of any particular chemical species estimated in the whole proteins could be accounted for in the extracted and residual proteins to within the random errors of analysis.

(6) *Extraction tests with* Festuca ovina

A closer examination of the natures and reactions of the morphological units of the leaf cells was demanded by the findings of this earlier work, and a series of experiments was designed accordingly.

The proteins of plants are almost entirely intracellular, and in living, somatic cells appear to occur mostly in the protoplasm: only in ripening and drying seeds has much protein been found in the vacuoles. The protoplasmic protein is distributed between the nuclei, the ground mass of the cytoplasm and the more solid particles such as the plastids and mitochondria (or chondriosomes). Crystalline and amorphous protein particles may be present free in the vacuoles and cytoplasm, and embedded in the nuclei and plastids. The possibility cannot be ignored that the protein or proteins coming into each of these categories may differ from those in any other, and there may be differences as between one cell and another. Menke [1938], extending the work of Noack [1927], has shown that nuclei and chloroplasts may be separated from the rest of the protoplasm fairly readily by centrifuging and by other means; and Fauré-Fremiet [1913] found that mitochondria could be removed by centrifuging. The protoplasm is thus to be regarded as consisting of fluid and more solid fractions but no simple embracing terms are in use in reference to the entire, mixed cell contents. For simplification of subsequent descriptions and discussions, the terms "non-granule" will be applied to the mixed liquid fractions of vacuoles and protoplasm, and "granule" to the nuclei, plastids, mitochondria, membranes etc.

As a solvent for the protein contained in chloroplast material which had been extracted with alcohol and ether, Menke [1938] employed a reagent used by Osborne *et al.* [1921] and later by Davies [1926], namely, 0·075 *N* NaOH in 60% alcohol–40% water solution. It is probable that the nitrogen extracted from leaf residues by the earlier workers with this reagent was largely of chloroplast origin. Menke [1938] sometimes used dilute aqueous NaOH solutions as solvents for leaf proteins, and they have been used also by Kiesel *et al.* [1934] and by earlier workers. These strongly alkaline reagents, however, could scarcely be expected not to degrade any protein dissolved in them (see e.g. Blish & Sandstedt [1929–1930] and Neglia *et al.* [1938]).

In the earlier experiments with *Festuca ovina*, it was found that by macerating the leaves very finely in a runner-mill with water, or better with a *p*H 7 or 9 buffer solution, and then centrifuging the mash for a short time in low-intensity fields, the forms of leaf N (total, coagulable and protein) were distributed through the fluid in macro-uniformity and almost in the proportions occurring in the leaves, and very little unextracted N remained in the particles of cell debris which, together with some of the fluid, formed the lower layer of loose sludge. It was otherwise if any attempt were made to separate the debris by pressing the mash in a cloth, for the debris itself acted as a filter to retain preferentially some of the coagulable and protein N, an effect which could be correlated with a visible retention of chloroplast material. From the granules separated by centrifuging in high-intensity fields, it was found possible to extract protein by emulsifying with quite mildly alkaline buffers and adding ether, alcohol and alcohol-ether mixtures; but if ether were ever present in such quantity as to permit a separation of liquid layers the chloroplast material was largely carried up with the ether and the protein in it rendered rather insoluble in the buffer, presumably by denaturation. Furthermore, if very large proportions of alcohol were added the buffer solutions failed to be satisfactory protein solvents.

Typical later experiments with *Festuca ovina* leaves are described in Table II with its accompanying explanation. Of particular interest is the fact that the alcohol-ether-*p*H 9·2 buffer mixture proved to be almost as effective in dissolving protein from the granules as was the alcohol-NaOH solution mixture. It disintegrated the granules (or at least the chloroplasts), the residues of which retained almost all their chlorophyll and apparently most of their lipin and centrifuged down much more cohesively than undisintegrated granules do. Microscopically, the disintegration was correlated with a tendency of the chloroplasts to lose their shape, to stick together and to smear on glass. From suspension in the buffers themselves, the denser nuclei centrifuged down as a small cream-coloured layer beneath the centrifuged chloroplasts, very much as they did from suspension in water [cf. Menke, 1938], but when the lipoid solvents were included with the buffers and the disintegration was pronounced, the cream-coloured layer could not be distinguished. The nuclei and mitochondria may have suffered a fate similar to that of the chloroplasts or they may have been carried down almost intact with the disintegrated chloroplasts. Some protein was obstinately retained by the disintegrated granule residues and it is not known to what morphological units it belonged, though it would probably have included any denatured protein, such as might have been present in dead cells.

Inasmuch as some N could be extracted from the granules without the aid of lipoid solvents, it would seem that they might yield much of their protein to water or to plain mildly alkaline buffer solutions if they could be disrupted mechanically to a sufficient degree. It must have occurred to some extent during the grinding operations which yielded the juices examined.

As to the nature of leaf-cell nucleoproteins, the work of Belozersky & Chigirev [1936] and Belozersky & Dubrovskaya [1936] suggests that the protein parts proper are unlikely to be of strongly basic character like the protamines and histones.

Exp. 6 (iv) showed that the granule disintegration could be carried out in the presence of the rest of the protoplasmic and vacuole material. From a comparison of the N contents of the relevant protein micro-preparations, it seemed too that the lipoid solvents could change the state of hydration or aggregation of certain substances in the juice (mucilages?) in such a way that they could be removed in part with the disintegrated granules by centrifuging in high-intensity fields, thus

reducing the extent to which they were able later to contaminate the flocculated protein. It will be noted that the proteins were more completely flocculated and coagulated from their solutions if alcohol was present, some of the extra "protein" obtained possibly being of proteose or similar nature.

(7) *Large-scale coarse-maceration extraction of* Lolium perenne

The presence of a mildly alkaline buffer during maceration of the leaves is favoured by the considerations that a fairly satisfactory solvent for solid (deposit) protein is thereby provided, that the other units comprising the granule fraction of the cell contents appear to suspend or emulsify in it better than they do in more neutral media, and that leaves which have so strongly acid a cell-sap that they fail to yield even their non-granule protein in solution when macerated with water might be expected to behave towards the buffer much as the *Festuca ovina* leaves behaved (cf. Chibnall & Grover [1926], who used dilute NaOH solutions to extract such leaves). In regard to the alkalinity of the buffer, the reversible-dissociation ranges of the proteins concerned should not be exceeded. It might be safe to use buffers at *p*H 10 or even *p*H 11. At all events, such evidence as does exist suggests that irreversible dissociation does not normally occur with proteins in the neighbourhood of *p*H 9, in which region the degree of reversible dissociation is frequently negligible (see e.g. Svedberg & Sjögren [1930, 1, 2]; Sjögren & Svedberg [1930, 1, 2]; and a recent review by Svedberg [1937]). Subsequent addition of lipoid solvents would decrease the ionization constants of buffer acids (thereby normally increasing the *p*H, but on a different *p*H scale) and the dissociation and ionization constants of the proteins would probably be modified too, but the resultant effects in regard to protein dissociation are not known.

The above considerations apply whether the leaf material is macerated finely or coarsely. By repeated fine maceration and avoidance of any loss of the granule fraction it would seem from experiment 6 that almost all the protein could be extracted and obtained as a preparation which might not be very pure but must be almost perfectly representative, and that at some sacrifice of representativeness purer preparations could be obtained with the aid of lipoid solvents. The large-scale extraction of leaf-protein samples, however, can be effected more easily by coarse than by fine maceration procedure. Many of the leaf cells are not ruptured by coarse maceration and those that are ruptured may not yield their contents to the juice in proper proportion if the maceration procedure itself involves a partial filtration by extrusion of the contents through split cell-walls. In principle, therefore, coarse maceration should amount to random clean cutting or tearing, without compression, and the requirements are fulfilled to a degree by a mincing-machine though not perfectly. *Lolium perenne* was one grass from which it had been lamentably easy to extract unrepresentative protein and it was accordingly chosen to provide fairly rigorous tests of procedures based upon the findings of Exp. 6 and adapted to large-scale extraction with the aid of the mincing-machine.

The leaf material was of medium age and had been grown in a rather shady environment. By finely macerating a small portion with a *p*H 9 buffer and centrifuging the juice as in Exp. 6, 32·8% of the leaf total N was found to be associated with the granule fraction containing all the visible chlorophyll. The main portion was passed through the mincing machine with three times its weight of a *p*H 9·2 sodium borate buffer containing 1·1 g. B per litre, the juice was separated from cell debris by violent shaking and stirring on a wire sieve (holes 1 mm. sq.) followed by centrifuging for 2 min. in a field of 100 × gravity,

and the residues were washed by stirring up with more buffer. The mixed juices were adjusted to *p*H 9·2 and divided up to give preparations 7E, 7E1, 7E2 and 7RE1 in Table I, 7RE1 representing disintegrated granule residue separated by the centrifuge before flocculating 7E1. The data have been calculated back as if all the juice had been used for each preparation. It should be noted that 7R, 7E1 and 7RE1 together contained virtually all the protein N calculated to have been present in the fresh leaves. 7E not having been flocculated and coagulated in presence of alcohol, some protein N (calculable from 7E1 and 7RE1 as about 10%) remained in solution in the extract, and gives the impression that much more non-coagulable N in proportion to coagulable N than was actually the case was extracted from the leaves. So too in the earlier extractions (Exps. 1, 2, 3, 4. and 5) must the proportions have been rather less unfavourable than they seemed. In the case of 7E2 there was considerable retention of protein N (and coagulable N) by the filter-pad but not much retention of non-coagulable N. It is one of the purest preparations which have been made from leaves of this grass. Whilst 7E might have been expected to contain too little of the non-granule protein, the reverse might have been expected of 7E1.

The whole, extracted and residual protein preparations (except 7RE1) were analysed, and the results are reported in Table III under 7W, 7E, 7E1, 7E2 and 7R. They showed, to within the limits of the random errors of analyses, no evidence of unrepresentative extraction of protein in any of the three preparations obtained: a most gratifying result in view of the hypothetical difficulties discussed. Apparently the large amount of protein retained by the filter-pad in the preparation of 7E2 was itself moderately representative of the whole protein.

(8) *Small-scale fine-maceration extraction of* Dactylis glomerata

Experiments were made with *Dactylis glomerata* with the object of generalizing further, if possible, some of the findings made with *Festuca ovina* (6) and *Lolium perenne* (7), and at the same time of obtaining high-efficiency extraction of the protein on a larger scale than in Exp. 6. 100 g. of fresh leaves of young-medium age, estimated by the method used in Exps. 6 and 7 to contain 33·1% of the total N in the granule fraction, were macerated in several portions in the runner-mill with a *p*H 9·2 borate buffer and the juice was separated from the residues with the aid of the sieve and the centrifuge as in Exp. 7, the residues being treated similarly twice in succession. The mixed juices, occupying a volume of nearly 1 litre, were adjusted to *p*H 9·2 and divided up for the preparation of 8E1, 8E2, 8E3, 8E4 (with 8RE4) and 8E5 as shown in Table I, the data being calculated back as if the entire amount of juice had been used for each preparation. 8RE4 represents the disintegrated granule material separated by the centrifuge before flocculating 8E4.

In general the results confirmed those found with the other plant species in Exps. 6 and 7. Again it was seen that the flocculation and coagulation of the protein in the juice were assisted by the presence of alcohol. In fact the recoveries of the whole (8W) coagulable N and protein N between the extracted and residual material, 8E2 and 8R or 8E4, 8RE4 and 8R, were a little in excess of 100%, and suggested that in drying the leaf material at 80° some 2% of the protein N had been converted into non-coagulable N. Just as in Exp. 6, centrifuging in a field of 500 × gravity (8E4) was less effective in preferentially removing contaminating substances from the juice containing alcohol and ether than filtration (8E5) was, but a field of 25,000 × gravity might have been as effective had it been possible to employ the high-speed centrifuge with such quantities of juice. Much protein was retained by the filter-pads whether alcohol and ether were

present (8E5) or not (8E3), but the former preparation, which appears to be the purest yet made from leaves of this grass, was considerably purer than the latter and was, presumably, more nearly representative of the whole leaf-protein. 8E2 must have been highly representative, whilst 8E1 may have been slightly deficient in the non-granule fraction. Of the total N in the residue 8R, rather too small a fraction in comparison with that of the original leaf material, was non-coagulable, suggesting that the residue contained insufficient non-granule protein to be representative of the whole. On the other hand it contained only 8·4% of the original protein N.

It is presumed that preparations similar to 8E4 and 8E5 could have been obtained by macerating the leaves in the presence of the lipoid solvents, but the general effects of having lipoid solvents present at this stage were not investigated.

Further considerations

Local acidity, causing differential flocculation of some of the non-granule proteins, is the only obvious hypothesis to be advanced in explanation of the rather pronounced differences in composition between 3E(*a*) and 3E(*b*). In passing it should be mentioned too that at *p*H values below 6 the non-granule proteins are usually very prone to surface denaturation. In the absence of salt their solutions in dilute HCl on the acid side of the isoelectric regions may frequently be boiled without appreciable coagulation.

It is of interest to compare the partial compositions of the whole proteins of the three batches of *Lolium perenne* leaves (3W, 4W and 7W), and those of two batches of *Dactylis glomerata* leaves (1W and 2W). The differences are small and of the most doubtful significance. In the first place, age of the leaves and climatic and manurial conditions and locality of growth appear to have been without appreciable effect upon composition of the whole leaf-proteins. In the second place there is no evidence that the whole leaf-proteins of the two species from the order *Gramineae* differed in composition. The whole leaf-protein of the single batch of *Beta cicla* from the order *Chenopodiaceae*, on the other hand, differed from these in having a lower tryptophan and a higher tyrosine content.

Even when appropriate precautions were not taken during extraction and the extracted proteins were demonstrably unrepresentative of the whole leaf-proteins, the differences in composition were not very great. Previously reported partial compositions of extracted protein preparations [Lugg, 1938, 2, 3] are therefore unlikely to have been seriously different from those of the whole proteins of the leaves from which they were obtained.

Summary

Samples of protein extracted from plant leaves in various ways have been examined for representativeness. Points favouring the use of mildly alkaline buffers, when macerating leaves with the object of dispersing the protein-containing cell-units into the juice, and the difficulties of avoiding preferential loss of the proteins belonging to the "granular" units (nuclei, plastids, mitochondria etc.) when subsequently removing cell-wall material and other impurities, have been examined and discussed. It has been shown that the addition of lipoid solvents, such as alcohol and ether, to the mildly alkaline juice permits most of the protein of the granule fraction to pass into solution; and the use of such solvents in the preparation of purer samples of protein without serious sacrifice of representativeness, has been demonstrated. Incidentally, the proteins

are much more completely flocculated by acid in their isoelectric regions and coagulated by heat, if alcohol is present in the solutions containing them.

The amide, tyrosine and tryptophan contents and the S distributions (cystine plus cysteine and methionine contents) of the whole proteins of leaves, provided no evidence of variation in composition with the age of the leaves or the manurial and climatic conditions or locality of growth; but composition may vary with plant species.

The continued valuable co-operation of Prof. A. C. Chibnall has been greatly appreciated. Thanks are due also to Dr K. Bailey and Dr G. R. Tristram, to Mr G. A. Stroud for much assistance, and to the Agricultural Research Council for providing a grant.

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XIV. THE ACTIVE PRINCIPLES OF *CANNABIS INDICA* RESIN. I

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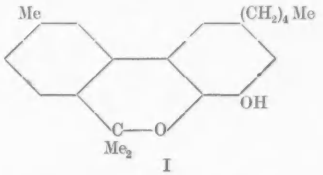
(*Received 24 November 1938*)

The resinous exudate of the female flowers of *Cannabis indica* (*C. sativa*) forms the essential constituent of the drug variously known as hashish, bhang, charas, ganja and marihuana according to the country of origin and mode of preparation. Extracts of the flowers have been used in European medicine to some extent but were found to be so variable in activity that *C. indica* has been removed from the *British Pharmacopoeia*. The plant is, however, well known through its use as a drug in oriental countries and recently, under the name of "marihuana", its use has assumed dangerous proportions in America. *Cannabis* resin has been the subject of many investigations in the past but much of the earlier work is contradictory. The active principle is contained in a high-boiling resin and is not an alkaloid [Smith, 1857]. The active fraction isolated from the crude resin by Wood Spivey & Easterfield [1896] and given by them the name cannabinol was later found by the same workers [1899] to be a mixture, and the name cannabinol was transferred to that portion of the active resin yielding a crystalline acetate, m.p. 75°. A good deal of confusion was introduced by later workers, who, although failing to obtain a crystalline acetate, nevertheless applied the name cannabinol to their products. The situation was largely clarified by Cahn [1931], who confirmed the observations of Wood Spivey & Easterfield and established for cannabinol the formula $C_{21}H_{26}O_2$. The constitution of cannabinol has been investigated by Cahn [1930–33] and by Bergel [1932], and for it the former proposed structure (I) in which only the positions of the hydroxyl and *n*-amyl groups are to be regarded as uncertain.



I

In his last communication Cahn states that "cannabinol is not the pharmacologically active principle of *Cannabis indica*" [1933]. It seemed desirable that further investigations on the resin should be made and we have taken up the study of cannabis resin with a view to isolating the substance or substances responsible for its pharmacological action.

The starting point in these investigations was material which corresponded to the high-boiling resin (b.p. 265°/20 mm.) first described by Wood Spivey & Easterfield [1896]. It was found that cannabinol can be removed almost quantitatively as its crystalline p-*nitrobenzoate* (amounting to 25% of the whole) on

p-nitrobenzoylating the resin. On hydrolysis the crystalline ester yields cannabinol as a colourless oil, which gives the above-mentioned crystalline acetate (M.P. 75°) on acetylation. The non-crystalline portion of the *p*-nitrobenzoylated resin gave on hydrolysis a colourless oil from which no crystalline acetate could be obtained.

Active hashish preparations induce a characteristic cataleptic condition in dogs, but this effect, described first by Fraenkel [1903] is not readily made the basis of quantitative assay. Gayer [1928] showed that in various animals—e.g. cats, rabbits, mice—hashish preparations induce corneal anaesthesia and that this effect is characteristic of active fractions of the resin. This Gayer test has been developed by Marx & Eckhardt [1933] using rabbits and has been employed throughout the work described in this paper. The pure cannabinol prepared from the crystalline *p*-nitrobenzoate was found to be highly toxic when injected intravenously into rabbits but, unlike the original resin, did not produce corneal anaesthesia. The cannabinol-free resin on the other hand was much less toxic and always induced corneal anaesthesia in rabbits. This distinction is further emphasized by the fact that an acetone solution of cannabinol was found to be non-toxic after standing for 3 days in contact with air while only slight loss of activity occurred on similar treatment of the cannabinol-free resin.

Attempts to fractionate further the cannabinol-free resin by distillation or crystallization of derivatives failed completely but chromatographic analysis gave promising results. Using activated aluminium oxide as adsorbent it was possible to obtain fairly readily an oil having considerably greater activity in the Gayer test than the starting material. Even better results were obtained by submitting the oily *p*-nitrobenzoate mixture left after separating the cannabinol *p*-nitrobenzoate to chromatographic analysis on activated aluminium oxide. By this procedure remaining traces of cannabinol were removed and an oil was obtained yielding on hydrolysis a product giving a positive result in the Gayer test at a dose of 0·25 mg. per kg. body weight; this material has low toxicity as compared with cannabinol and possesses none of the convulsant action of the latter substance.

Further investigation of this highly active material is in progress and the results will be reported later. We have not as yet obtained any crystalline derivative from the most active preparation and suspect that it does not yet represent the homogeneous active principle.

Experimental

Starting material. The starting material was the resin (B.P. 185–190°/0·6 mm.) obtained on working up hashish of Indian origin in the manner described by Bergel [1930]. The resin was nearly colourless when freshly distilled. It corresponds to the "crude cannabinol" of Wood Spivey & Easterfield and possessed the characteristic pharmacological properties of the original extract.

Isolation of cannabinol. The above resin (25 g.) was dissolved in pyridine (110 ml.) and *p*-nitrobenzoyl chloride (36 g.) added. The mixture was refluxed for 4 hr., then poured on a mixture of ice and sufficient H_2SO_4 to make the resulting suspension acid to Congo red. The precipitate was collected, washed with water, dried and refluxed with light petroleum (750 ml., B.P. 80–100°) for 1 hr. and filtered hot, the filter residue being treated in the same way with a further quantity of light petroleum (250 ml.). The combined filtrates were washed with aqueous Na_2CO_3, dried and concentrated to about 150 ml. On standing *cannabinol* p-*nitrobenzoate* separated. Recrystallized first from alcohol

then from light petroleum (B.P. 80–100°) it formed pale yellow needles M.P. 160° (yield, *ca.* 6·5 g.). (Found: C, 73·0; H, 6·2; N, 3·2 %. $C_{28}H_{29}O_5N$ requires C, 73·2; H, 6·3; N, 3·2 %.) Since cannabinol *p*-nitrobenzoate is very sparingly soluble in methyl alcohol the oil left on evaporating the original light petroleum mother liquors may be largely freed of cannabinol by fractionation with this solvent.

Cannabinol p-*aminobenzoate.* Cannabinol *p*-nitrobenzoate (1 g.) dissolved in alcohol (80 ml.) was hydrogenated using a platinum oxide catalyst. Absorption of hydrogen ceased when 200 ml. had been absorbed (theoretical 190 ml.). The resulting p-*aminobenzoate* crystallized from methyl alcohol in colourless needles M.P. 149–150°. (Found: C, 78·2; H, 7·4 %. $C_{28}H_{31}O_3N$ requires C, 78·3; H, 7·2 %.)

Cannabinol. Cannabinol *p*-nitrobenzoate hydrolysed by refluxing with methyl alcoholic KOH (5 %) during 1½ hr. gave cannabinol as an almost colourless oil which, with acetic anhydride-pyridine gave in quantitative yield a crystalline acetate M.P. 75° not depressed on admixture with a sample of cannabinol acetate kindly supplied by Dr R. S. Cahn.

Pharmacological tests

The Gayer test was carried out on rabbits as described by Marx & Eckhardt [1933], the substances being injected in acetone solution (0·5 % wt./vol.).

(*a*) *Cannabinol.* Pure cannabinol prepared by hydrolysing the *p*-nitrobenzoate and subsequent distillation in a high vacuum was used. In doses less than 2 mg. per kg. body weight the material had no visible effect and the corneal reflex remained normal. At any higher dosage the following sequence of events was observed. For about 1 min. the animal behaved normally, but at the end of this time it lay down and in a few seconds rolled over on its side and became rigid, the corneal reflex remaining normal. In any time from a few seconds to 1 min. later, depending on the size of the dose, the rabbit went into violent convulsions terminating in death within about 30 sec.

(*b*) *Material from non-crystalline* p-*nitrobenzoates.* After separation of the crystalline *p*-nitrobenzoate the mixture of oily esters from the resin was hydrolysed and the product distilled in a high vacuum. The nearly colourless oil obtained had no effect when injected into rabbits in doses less than 1 mg. per kg. body weight. In a dose of 1 mg. per kg. body weight the drug had no effect for about 3 min., after which time the animal's head began to nod gently and it subsided to its normal sleeping position and remained so. During this period the corneal reflex slowly disappeared until no response could be elicited. The animal could be roused for a few seconds by violent shaking but when left undisturbed quickly relapsed. In doses up to 5 mg. per kg. body weight exactly the same effect was observed, the period of sleep or stupor extending from 30 min. up to 6 or 7 hr. after which the rabbit recovered completely. In doses of 5 mg. or more the animal, although giving a positive Gayer test, died with convulsions within the first 2 hr. after injection. The death after convulsions was presumably due to the presence of some cannabinol in the injected material (cf. below).

Chromatographic analysis of hydrolysate of non-crystalline p-*nitrobenzoates*

A sample of the oil (2 g.) prepared by hydrolysis of the non-crystalline fraction of the *p*-nitrobenzoylated resin and subsequent distillation in a high vacuum was dissolved in light petroleum (300 ml.; B.P. 60–80°) and allowed to percolate through a column of activated aluminium oxide (Merck), the chromatogram being developed first with light petroleum (750 ml.; B.P. 60–80°), then with a mixture (1 litre) of equal parts of light petroleum (B.P. 60–80°) and ether.

When the column was viewed in ultraviolet light four distinct bands were visible; from the top downwards these were: (1) yellow 6 cm., (2) colourless 8 cm., (3) yellow 8 cm., (4) blue fluorescent 6 cm. On elution with a mixture of ether and methyl alcohol (4 : 1) the oils from sections (1), (2) and (3) were found to be inactive when tested on rabbits while that from section (4) gave a positive Gayer test in a dose of 3 mg. per kg. body weight.

The oil from section (4) was therefore combined with that obtained by evaporating the filtrate from the chromatogram, dissolved in light petroleum (B.P. 60–80°) containing 5 % ether and re-adsorbed on a column (2 × 30 cm.) of activated aluminium oxide (Merck), the chromatogram being developed with the same solvent mixture. From the top downwards the column showed in ultraviolet light the following bands: (1) purple 3 cm., (2) yellowish blue 10 cm., (3) deep blue 10 cm., (4) strongly blue fluorescent 2 cm., (5) yellow 3 cm. Tests on rabbits showed that the oil from section (4) (50 mg.) was lethal in a dose of 2·5 mg. per kg. body weight and had the typical action of cannabinol. The oil from section (2) (60 mg.) was active in the Gayer test in a dose of 1 mg. per kg. body weight and when injected in a dose of 5 mg. per kg. body weight the animal survived for 10 hr. without any trace of muscular rigidity and died in sleep without any convulsions. The oil from section (3), like the starting material, possessed both convulsant and sleep-producing properties.

Chromatographic analysis of non-crystalline p-*nitrobenzoates.* The oily residue (8 g.) left on removing the crystalline cannabinol derivative from the *p*-nitrobenzoylation product of the distilled resin, was dissolved in light petroleum (B.P. 40–60°) and subjected to adsorption on a column (5 × 45 cm.) of activated aluminium oxide (Merck) previously washed with a solution of phenol in light petroleum to reduce alkalinity. After developing with light petroleum (4 litres) the chromatogram showed six distinct bands when viewed in ultra-violet light. Each of these was separately eluted, hydrolysed, distilled and tested, the filtrate from the column being evaporated and the residue similarly treated and tested. The following table shows the results obtained, the bands in the chromatogram being numbered from the top downwards.

	Colour	Length cm.	Wt. of eluate g.	Millon test	Gayer test	M.L.D. mg.
1	Yellow	3	0·3	–	–	–
2	Colourless	15	1·2	–	–	–
3	Bright blue	3	0·25	+	–	6
4	Colourless	10	2·0	+	–	5·5
5	Yellow	5	0·9	–	–	–
6	Colourless	10	1·2	+	+(2 mg.)	9
7	Colourless	Filtrate	1·5	+	+(0·25 mg.)	5

The biological test results were reproducible in different animals, there being very little variation either in the minimum active dose or in the minimum lethal dose (M.L.D.). Sections (3) and (4) possessed the typical convulsion-producing properties of cannabinol while sections (6) and (7) had no such action. It is clear then from the table that the remaining traces of cannabinol were concentrated in sections (3) and (4), while the material producing corneal anaesthesia was concentrated in sections (6) and (7). The oil from sections (6) and (7) gave, like cannabinol, a precipitate with Millon's reagent.

Relative stabilities of cannabinol and resin from fraction 7 (*above*). A solution (0·5 %) of cannabinol in acetone was exposed to the air for 3 days. When tested on rabbits at the end of this time it was found to be non-toxic. A solution of the

resin from fraction 7 (above) after standing for 6 months under the same conditions retained about 25 % of its activity in the Gayer test.

SUMMARY

p-Nitrobenzoylation of the high-boiling pharmacologically active resin from the female flowers of *Cannabis indica* yields crystalline cannabinol *p*-nitrobenzoate and a mixture of resinous esters. Cannabinol is highly toxic and gives a completely negative reaction in the Gayer hashish test on rabbits, while the hydrolysis product of the resinous esters gives a strong positive reaction and is less toxic than cannabinol. The material giving a positive Gayer test has been fractionated by adsorption methods and a product obtained showing a positive Gayer test in rabbits in a dose of 0·25 mg. per kg. body weight.

One of the authors (F. B.) desires to record his appreciation of the hospitality accorded him in the University College and the Laboratory of the Public Analyst, Colombo, and to thank the Director of the Royal Botanic Gardens, Peradeniya, Ceylon, for his generous assistance.

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XV. THE RELATIONSHIP OF CARBOHYDRATE METABOLISM TO PROTEIN METABOLISM

I. THE ROLES OF TOTAL DIETARY CARBOHYDRATE AND OF SURFEIT CARBOHYDRATE IN PROTEIN METABOLISM

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Earlier observations showed that the provision of excess of energy in the human diet is attended by a storage of N and S, the storage being more marked when the surfeit is in the form of carbohydrate than when in the form of fat [Cuthbertson & Munro, 1937]. Simultaneously with these observations, it was demonstrated by Larson & Chaikoff [1937] in one-day superimposition experiments on dogs, that this storage was only provoked by carbohydrate ingested within 4 hr. before or after the protein meal. Moreover, the closer the proximity in time of the surfeit carbohydrate to the meal, the greater was the quantity of N stored.

These effects of surfeit carbohydrate on protein metabolism prompted us to investigate the function of the total carbohydrate of the ordinary adequate diet, as it affects N metabolism, especially with regard to the relative times of ingestion of the protein and the carbohydrate. The present investigation of protein metabolism is divided into four sections:

(I) The effect of complete separation as regards time of ingestion of all, or practically all, the carbohydrate and the protein of an adequate diet (Exps. 1–5).

(II) The effect of separation of only a part of the protein from the carbohydrate in such a diet (Exp. 6).

(III) The effect of taking surfeit carbohydrate at a time separated from that of the ingestion of the protein (Exp. 7).

(IV) The effect on N balance of varying the number of meals in a fixed diet (Exp. 8). (This is intended as a control for certain aspects of the other experiments.)

Experimental

The analytical procedures were similar to those referred to in a previous paper [Cuthbertson & Munro, 1937]. The fluid intake was kept as constant as possible throughout these experiments.

I. *Separate ingestion of dietary protein and carbohydrate*

Exps. 1 and 2. In preliminary experiments, two adults, A. H. S. and H. B. C. (Table I), were put on diets adequate for maintenance, containing 80 g. protein and 2740 cal. in the case of the former, and 97 g. and 3400 cal. in the case of the latter. During Period I this food was divided for both subjects into four equal mixed meals, taken at 8.30 a.m., 1 p.m., 4.30 p.m. and 10 p.m. respectively (Tables II and III). Their body-weights remained constant under this regime.

Table I. *The experimental subjects*

Subject	Age yr.	Height cm.	Wt. kg.	Exp. no.	Diet: Protein g.	Diet: Carbohydrate g.	Diet: Fat g.	Diet: Calories
A. H. S.	30	168	64	1	80	367	101	2740
H. B. C.	24	184	80	2	97	455	127	3400
H. N. M.	22	164	61·5	3	84	400	61	2500
			61·25	6	116	390	87	2880
			64·4	7	76	320	140	2930
			63·9	8	76	320	140	2930
D. P. C.	38	184	83	4	66	408	172	3500
			83·7	5	76	408	174	3560

Moreover, if one allows 1·3 g. as covering the daily faecal and cutaneous losses of N (which were not estimated), the average daily urinary N values for this period (10·82 g. for A. H. S.; 14·27 g. for H. B. C.) agree well with their N intakes of 12·56 and 15·5 g. respectively, confirming the attainment of N equilibrium in each subject.

In Period II, this arrangement of meals was altered so that all the protein and a part of the fat of the diet were divided between the meals at 8.30 a.m. and 4.30 p.m., while the remainder of the fat and practically all the carbohydrate were consumed at 1 p.m. and 10 p.m. (Tables II and III). This almost complete separation of protein from carbohydrate resulted in a marked loss of N, which obtained throughout the period of observation and amounted on the average to 1·77 g. daily for A. H. S. and 2·02 g. for H. B. C. (Figs. 1 and 2). It will be noticed that during no part of the day was there any lack of energy-yielding food material (Tables II and III).

In the case of H. B. C. a return to the previous dietary was made in Period III, and this effected an immediate cessation of the N loss. Under the arrangement of meals in Period II the protein-containing meals were reduced in number from 4 to 2 (Tables II and III); that this procedure was not of itself productive of the marked negative N balance will be subsequently demonstrated in Exp. 8.

Exp. 3. This experiment followed along the lines of the previous two, but differed in that the procedure was reversed; the daily urinary collection was separated into two fractions, from 8 a.m.–8 p.m. and 8 p.m.–8 a.m., and account was taken of urea, preformed sulphate and creatinine as well as N excretions. The subject H. N. M. (Table I) partook of a diet containing egg-white, cheese, casein, arrowroot, sugar, butter and apples (84 g. protein, 61 g. fat and 400 g. carbohydrate, in all, some 2500 cal.).

In Period I, all the protein and part of the fat were taken at 9.30 a.m. and 12.45 p.m., while the rest of the fat and all the carbohydrate were spread between 4.30 and 7.30 p.m. (Table IV). During this period of separation of carbohydrate from protein, the daily N intake was 13·36 g., while the urinary output averaged 14·80 g. (Fig. 3), a considerable loss.

In Period II, the carbohydrate and fat of the previous two later meals were taken instead along with the protein and fat at 9.30 a.m. and 12.45 p.m.; the hours of protein ingestion were thus unchanged from Period I, whilst the carbohydrate was now associated with the protein. This produced an immediate fall in urinary N output, down to an average over the period of 12·67 g. (intake 13·36 g.), which is presumably only slightly in excess of N equilibrium. The difference in N output between Periods I and II amounted to 2·13 g. daily.

Table II. *Dietary of subject A. H. S., Exp. 1*

	Period I				Period II			
	8.20 a.m.	1 p.m.	4.30 p.m.	10 p.m.	8.30 a.m.	1 p.m.	4.30 p.m.	10 p.m.
	Same food at all meals (g.)				g.	g.	g.	g.
Lean beef	71·25				142·5	—	142·5	—
Egg	37·5				75	—	75	—
Lettuce	10				20	—	20	—
Apple	50				100	—	100	—
Butter	15				—	30	—	30
Arrowroot	35				—	70	—	70
Sugar	56				—	112	—	112
Calorie distribution at each meal:								
Protein	81				166	—	166	—
Carbohydrate	367				46	688	46	688
Fat	236				240	232	240	232

Table III. *Dietary of subject H. B. C., Exp. 2*

	Periods I and III				Period II			
	8.30 a.m.	1 p.m.	4.30 p.m.	10 p.m.	8.30 a.m.	1 p.m.	4.30 p.m.	10 p.m.
	Same food at all meals (g.)				g.	g.	g.	g.
Lean beef	85				170	—	170	—
Egg	50				100	—	100	—
Lettuce	10				20	—	20	—
Apple	50				100	—	100	—
Butter	15				—	30	—	30
Arrowroot	42				—	84	—	84
Sugar	71				—	142	—	142
Calorie distribution at each meal:								
Protein	100				200	—	200	—
Carbohydrate	455				46	864	46	864
Fat	296				360	232	360	232

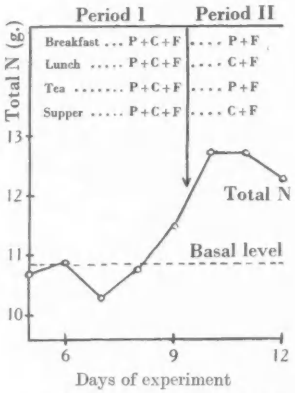

Fig. 1. Exp. 1. Subject A.H.S.

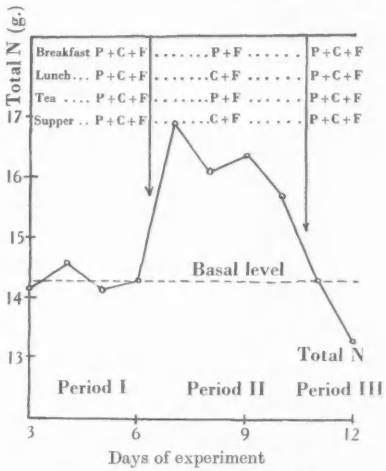

Fig. 2. Exp. 2. Subject H.B.C.

Figs. 1 and 2. Effect on urinary N excretion of separately ingesting the total protein and the carbohydrate of an adequate diet. P=protein. C=carbohydrate. F=fat.

Table IV. *Dietary of subject H. N. M., Exp. 3*

	Period I				Period II	
	9.30 a.m. cal.	12.45 p.m. cal.	4.30 p.m. cal.	7.30 p.m. cal.	9.30 a.m. cal.	12.45 p.m. cal.
Protein	172	172	—	—	172	172
Carbohydrate	—	—	820	820	820	820
Fat	116	116	168	168	284	284

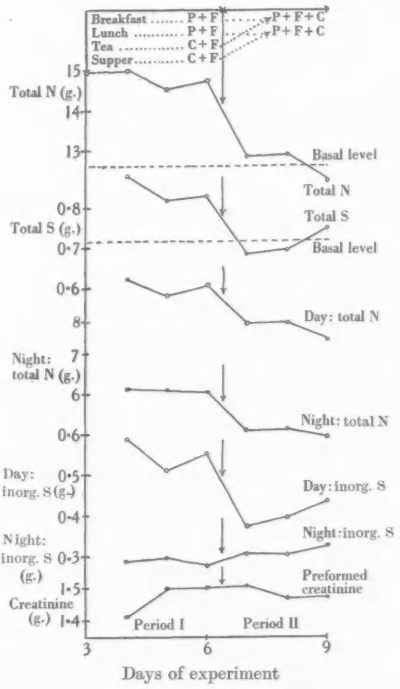

Fig. 3. Exp. 3. Subject H.N.M.

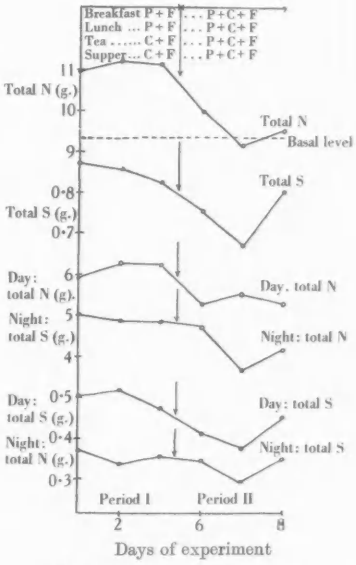

Fig. 4. Exp. 4. Subject D.P.C.

Figs. 3 and 4. Effect of separately ingesting the protein and carbohydrate of an adequate diet.

Urea and also NH_3 excretion closely paralleled that of total N. The preformed sulphate-S also took the same trend as the N, the excretion in Period I in excess of that in Period II averaging 0·129 g. S daily. The creatinine N output, however, remained relatively constant throughout. A study of the day (8 a.m.–8 p.m.) and night (8 p.m.–8 a.m.) urines indicates that the N content of each was equally affected by the change-over from Period I to Period II. On the other hand, only the day excretion of preformed sulphate was affected, the night excretion remaining relatively constant. It was not possible to record body-weights during the whole course of this experiment.

Exp. 4. This was confirmatory of the previous experiment. The subject D. P. C. (Table I) took a diet (Table V) containing 66 g. protein and 3500 calories,

which were so distributed as to enable him to eat meals consisting only of protein and fat at 8.10 a.m. and 1.15 p.m., the meals at 4.45 p.m. and 10.30 p.m. containing only carbohydrate and fat. During Period I the average daily urinary N output was 11·07 g. (Fig. 4), which with a faecal N of 1·29 g. daily (derived from Exp. 5) indicates a marked loss when compared with the intake of 10·52 g. N.

Table V. *Dietary of subject D. P. C., Exp. 4*

	Period I				Period II			
	8.10 a.m. g.	1.15 p.m. g.	4.45 p.m. g.	10.30 p.m. g.	8.10 a.m. g.	1.15 p.m. g.	4.45 p.m. g.	10.30 p.m. g.
Cheese	60	60	—	—	60	60	—	—
Butter	20	20	35	35	50	50	5	5
Egg	125	150	—	—	125	150	—	—
Lettuce	25	—	25	25	25	50	—	—
Apple	—	—	100	100	—	—	100	100
"Oxo"	—	½ cube	—	—	—	½ cube	—	—
Sugar	—	—	146	146	48	48	50	50
Arrowroot	—	—	60	60	60	60	60	60
Calorie distribution at each meal:								
Protein	130	140	—	—	130	140	—	—
Carbohydrate	—	—	816	816	384	384	432	432
Fat	493	521	293	293	493	521	38	38

The association in Period II of a considerable portion of the carbohydrate of the evening meals with the protein-containing meals of the earlier part of the day (Table V) resulted in a sharp drop in the N output to an average value of 9·27 g. daily on the last 2 days of this period (Fig. 4). N equilibrium was virtually obtained by this normal distribution of the food. The division of the urine into day (8 a.m.–8 p.m.) and night (8 p.m.–8 a.m.) portions showed that the fall in N was equally distributed between them. The total S output took a similar trend to the total N, the major change in it occurring in the day, however. These results are confirmatory of the findings in Exp. 3.

Exp. 5. A further experiment was also performed on D. P. C. (Table I), its main object being to determine whether this effect of carbohydrate in promoting the proper utilization of dietary protein were of a quantitative nature or not. A subsidiary consideration was the observation of the changes in body-weight over more prolonged periods of time than the previous separation experiments permitted, and under more uniform conditions.

The diet was similar to that of the preceding experiment (except that 25 g. diabetic bread was included) and contained some 3560 cals. and 76 g. protein; it was so disposed that protein and fat were the constituents of the meals at 8.10 a.m. and 1.15 p.m., and carbohydrate and fat of meals at 4.45 p.m. and 10.30 p.m. On this diet the urinary N averaged 12·80 g. for the 4th and 5th days of Period I (Table VI), the faecal N being 1·29 g. daily. If one allows 0·3 g. for cutaneous loss of N, there is thus a considerable negative N balance over the intake of 12·17 g. N.

On the 6th and 7th days (Period II) 25 g. cane sugar were transferred from the latter two meals of the day and associated with the protein meals at 8.10 a.m. and 1.15 p.m. (half to each meal). A definite reduction in the urinary output, averaging 0·91 g. N and 0·085 g. S daily, took place. In Period III a return to the regime of Period I produced an average daily urinary N over 4 days of 12·58 g.; excretion of S also rose. The transfer of 25 g. cane sugar was again made in

Table VI. *Exp. 5, subject D. P. C. The effect of separately ingesting the protein and carbohydrate in the diet and the effect of varying the amount of carbohydrate associated with the protein*

			Urine						
				N (g.)			S (g.)		
Period	Days of exp.	Body wt. kg.	Vol. ml.	Total	Day	Night	Total	Day	Night
I	1	83·70	—	—	—	—	—	—	—
	2	83·70	—	—	—	—	—	—	—
	3	83·68	2150	13·08	—	—	1·006	—	—
	4	83·68	2090	12·81	7·42	5·39	1·005	0·576	0·429
	5	83·50	1770	12·78	7·65	5·13	1·005	0·612	0·393
II	6	83·20	2210	12·02	6·84	5·17	0·952	0·589	0·363
	7	83·40	1970	11·76	6·57	5·185	0·889	0·532	0·357
III	8	83·50	2290	12·57	7·00	5·57	0·957	0·572	0·385
	9	82·95	2005	12·63	7·56	5·07	0·949	0·577	0·372
	10	82·85	2220	12·38	6·79	5·59	0·991	0·561	0·430
	11	82·80	1965	12·73	7·41	5·32	0·965	0·601	0·364
IV	12	82·75	2280	11·70	6·47	5·23	0·936	0·534	0·402
	13	82·85	2065	12·68	6·51	6·17	0·984	0·529	0·455
V	14	82·73	2180	12·58	7·11	5·47	0·917	0·512	0·405
VI	15	82·72	2270	11·81	7·14	4·67	0·893	0·558	0·335
	16	82·40	2300	11·79	6·72	5·07	0·861	0·488	0·373
	17	82·56	2200	11·54	6·72	4·82	0·893	0·522	0·371
VII	18	82·20	2575	11·71	6·63	5·08	0·861	0·504	0·357
	19	82·65	1770*	11·57	6·46	5·11	0·794	0·422	0·372
	20	82·67	2550	10·78	6·04	4·74	0·840	0·424	0·416

* Accidental loss of part of day urine. Figures inserted have been arrived at by analysis of the residual urine for N, S and creatinine and then by a calculation based on the constancy of the creatinine excretion in these experiments.

Diet

Period I. Meal 1 P+F 8.10 a.m. Meal 2 P+F 1.15 p.m. Meal 3 C+F 4.45 p.m. Meal 4 C+F 10.30 p.m.
Period II, as above, but 12·5 g. cane sugar added to meals 1 and 2 and removed from 3 and 4.
Period III, similar to I.
Period IV, as for I, but 25 g. cane sugar added to meal 2 and removed equally from 3 and 4.
Period V, similar to Period I.
Period VI, as for I, but 100 g. cane sugar added to meal 2 and removed equally from 3 and 4.
Period VII. Days 18 and 19 as for Period VI, but half cane sugar replaced by starch. In addition 100 g. carbohydrate were removed from meals 3 and 4 and taken at meal 1 as cane sugar. Meals 3 and 4 contained fat and equal amounts of starch and sugar. Day 20, as for preceding 2 days, but half sugar at breakfast replaced by starch.

Period IV, but on this occasion the depression was more transitory than in Period II.

In Period V a single day return to the dietary of Period I was made. Then in Period VI, 100 g. carbohydrate (as cane sugar for the first 2 days) were transferred from the evening meals to the protein meal at 1.15 p.m.; the resulting fall in N and S excretions averaged 0·87 g. and 0·084 g. respectively below the mean of Period III. On the last day of the period starch was substituted for half of the cane sugar, but this did not appreciably affect the N and S excretions.

During the last 3 days of the experiment further slight changes were made in the distribution of the diet, as indicated in Table VI. In the end, loss of body-weight was stopped and N equilibrium was reached at a level 2·02 g. lower than the original level of Period I, and 1·80 g. lower than the intermediate value of Period III. The S excretion also decreased during the final period; its unsteady nature, however, prohibited calculation of the exact amount of the fall. In this experiment the main changes in N and S were confined to the day urines.

II. *Partial separation of protein from carbohydrate*

Exp. 6. The possibility that separation of a part only of the protein from the dietary carbohydrate might also result in a loss of N from the body is considered in this experiment. The subject H. N. M. (Table I) was put on a diet containing some 2880 cal. and 116 g. protein, arranged (Table VII) so that he partook in Period I of mixed meals (each containing 2·32 g. N) at 9.30 a.m. and 8 p.m.; along with these meals a protein-free jelly of arrowroot and sugar (480 cal. per meal) was consumed. In the intervening hours of the day, 200 g. lean beef were taken at 12.45 p.m. and at 4.30 p.m. respectively. Thus during this period, some 75% (14·2 g.) of the dietary N was unassociated with carbohydrate; the average daily urinary excretion was 16·57 g. N, representing a slight storage with the intake of 18·8 g. N, if an allowance of 1·3 g. N for faecal and cutaneous loses is made (Table VIII).

Table VII. *Dietary of subject H. N. M., Exp. 6*

	Period I				Period II			
	9.30 a.m. g.	12.45 p.m. g.	4.30 p.m. g.	8 p.m. g.	9.30 a.m. g.	12.45 p.m. g.	4.30 p.m. g.	8 p.m. g.
White bread	81	—	—	81	81	—	—	81
Brown bread	42·5	—	—	42·5	42·5	—	—	42·5
Cheese	3·75	—	—	3·75	3·75	—	—	3·75
Bran	3·75	—	—	3·75	3·75	—	—	3·75
Jam	25	—	—	25	25	—	—	25
Butter	30	—	—	30	30	—	—	30
Apple	50	—	—	50	50	—	—	50
Lean beef	—	200	200	—	—	200	200	—
Arrowroot	45	—	—	45	—	45	45	—
Sugar	75	—	—	75	—	75	75	—
Total N	2·32	7·10	7·10	2·32	2·32	7·10	7·10	2·32
Carbohydrate	195	—	—	195	75	120	120	75

Table VIII. *Exp. 6, subject H. N. M. Partial separation of protein from carbohydrate*

Day of exp.	Diet		Body wt. kg.	Total urinary N output (g.)
2	Period I		61·35	16·39
3	(association of 25% of the protein		61·15	16·54
4	with carbohydrate)		61·26	17·19
5			61·20	16·19
6			61·25	16·21
7			61·10	16·88
		Mean	61·05	16·57
8	Period II		60·80	16·65
9	(association of 100% of the protein		60·90	16·20
10	with carbohydrate)		60·95	16·47
11			60·85	16·34
		Mean	60·90	16·42

In Period II the jelly of arrowroot was transferred to the meat meals at 12.45 p.m. and 4.30 p.m. The average daily output during this period was 16·42 g. N. Thus it may be said that, within the limits of our experiment, variation in the quantity of protein unassociated with carbohydrate in a diet does not affect the N balance, amounts as low as 4·64 g. N being sufficient, when ingested along with carbohydrate, to maintain N equilibrium.

III. *Separation of surfeit carbohydrate from dietary protein*

Exp. 7. This portion of the work was undertaken to investigate the function of carbohydrate eaten in excess of energy requirements under conditions similar

Table IX. *Dietary of subject H. N. M., Exps. 7 and 8*

	9.30 a.m.	12.45 p.m.	4.30 p.m.	7.15 p.m.
	g.	g.	g.	g.
White bread	50	150	100	25
Brown bread	25	60	60	25
Cheese	—	15	—	—
Bran	—	—	—	15
Apples	—	100	—	100
Butter		125 g.		
Jam		100 g.		
Milk	—	—	250 ml.	250 ml.

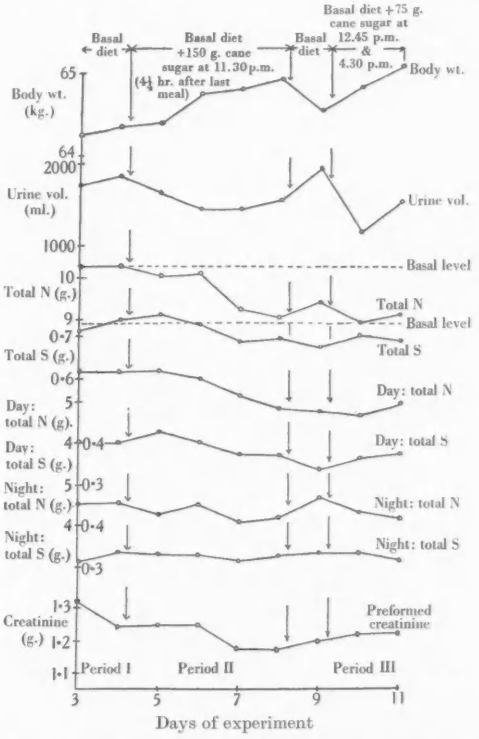

Fig. 5. Exp. 7. Subject H.N.M. Effect of varying the proximity of a carbohydrate surfeit to the rest of the diet.

to those in which the total dietary carbohydrate was ingested in the foregoing experiments, i.e. along with and also separate from the dietary protein. It will be remembered that the somewhat similar experiments of Larson & Chaikoff [1937]

were only one-day superimpositions of carbohydrate, and we wished to obtain evidence based on more prolonged surfeit feeding. The subject H. N. M. (Table I) was given a mixed diet (Table IX) containing 76 g. protein and some 2930 cal. In Period I urinary N equilibrium was established at a level of 10·32 g. with an intake of 12·10 g. (Fig. 5).

In Period II 150 g. sucrose were added daily at 11.30 p.m. ($4\frac{1}{4}$ hr. after the last meal). For 2 days this surfeit failed to induce any appreciable alteration in N output, but on the 3rd day of surfeit a sharp fall was registered and a new level of 9·14 g. N established. After an interval of one day, on which surfeit was discontinued, Period III was entered on by superimposing the 150 g. sucrose along with the 12.45 p.m. and 4.30 p.m. meals (the surfeit being divided between each) for 2 days. This maintained the N output at an average level of 9·04 g. per day.

An examination of the day (9.30 a.m.–9.30 p.m.) and night (9.30 p.m.–9.30 a.m.) urines shows that both in Period II and in Period III the major portion of the fall in N was attributable to the day fraction, no matter whether the carbohydrate were superimposed in the late evening or in the middle of the day. The fall in N was mainly due to a proportional reduction in urea excretion. In general, total S excretion paralleled total N, any changes noted in the level, however, being confined to the day fraction.

The creatinine output remained relatively constant throughout the course of the experiment. Body-weights were recorded and it will be observed that the giving of surfeit carbohydrate was accompanied by a rise in weight. This rise in weight in no way accounts for the marked and sustained reduction in urinary volume persisting throughout the periods of surfeit; probably this is to be explained by an increase in insensible perspiration, representing an attempt by increased energy metabolism to deal with the surfeit.

IV. *N balance in relation to the number of meals*

Exp. 8. Since in Exps. 1 and 2 the number of protein-containing meals was reduced from 4 to 2 in the second period of each, it was considered advisable to eliminate this change as a possible cause of the phenomena observed in these experiments (this objection does not apply to Exps. 3 and 4).

Table X. *Exp. 8, subject H. N. M., N balance in relation to the number of meals*

			Urine		Faeces	
Day of exp.	Diet	Body-wt. kg.	Volume ml.	N-output g.	N g.	Fat g.
4	Period I	64·05	1895	10·53		
4	(8 meals per day)	63·50	2238	10·89	Total	Total
5		63·95	1838	10·83	3·64	17·08
7		64·05	1915	10·72		
	Mean	63·89	—	10·74	0·91	4·27
8	Period II	63·60	2075	10·74		
9	(2 meals per day)	63·50	1650	11·83	Total	Total
10		64·15	1650	11·07	3·54	17·73
11		64·00	2155	11·00		
	Mean	63·81	—	11·16	0·89	4·43
12	Period III	63·90	2048	10·20	—	—
13	(8 meals per day)	63·95	1960	10·45	—	—
14		—	1990	10·75	—	—

The subject H. N. M. (Table I) was placed on a mixed diet similar to that of Exp. 7 (Table IX), but divided in this case into 8 equal meals separated by intervals of 2 hr. from one another. This gave a very constant N output averaging daily 10·74 g. in the urine and 0·91 g. in the faeces (intake 12·10 g. N). In Period II this diet was compressed into 2 equal meals, taken at 11 a.m. and 5 p.m. respectively. The effect on the N output was an evanescent rise (probably due to the alteration in the times of protein ingestion), the mean for all 4 days of this regime being only 11·16 g. N per day. The faecal fat and faecal N were unaltered by this change. In Period III the 8 meals were restored; this produced a temporary depression of N, followed by a return to the average output in Period I (Table X).

It may accordingly be said that since a change from 8 to 2 meals induces only a slight alteration in daily N output, the reduction of the protein-containing meals from 4 to 2 in Exps. 1 and 2 is at most only a minor factor, if indeed one at all, in the production of the marked losses of N observed therein.

Discussion

The salient features of our experiments may be grouped under the divisions suggested in the introductory remarks.

I. Exps. 1–4 indicate that the output of N and S from the body exceeds the intake when the adult human subject is fed on a diet abnormal only in one respect, viz. that the ingestion of the protein moiety is dissociated in time from that of the carbohydrate. This loss of N and S from the body occurs immediately on separating protein from carbohydrate and persists throughout the period of separation. In assessing the extent of this loss we have taken the mean daily level of urinary N output during the period of mixed meals as representing N equilibrium and have regarded as N lost from the body any excess output over and above this mean level. On such a basis, during the periods of separation of protein from carbohydrate the mean daily output of urinary N in our 4 experimental subjects was 1·93 g. greater than the N output when on mixed meals, i.e. there was a daily average of 1·93 g. N lost from the body. The individual data for these 4 subjects are appended in Table XI.

Table XI

		Material lost daily					
Exp.	Subject	N g.	S g.	N:S ratio	Body-wt. kg.	Loss per kg. wt. g. N	Loss per sq. metre surface area g. N
1	A. H. S.	1·77	—	—	64	0·025	0·94
2	H. B. C.	2·02	—	—	80	0·028	1·04
3	N. N. M.	2·13	0·129†	15·5:1*	61·5	0·035	1·29
4	D. P. C.	1·81	0·118	15·3:1	83·7	0·022	0·89

* Urea-N: preformed sulphate-S. † Preformed sulphate-S.

It will be seen that the losses of N vary between 1·77 and 2·13 g. daily for the different subjects. Age may be a factor in determining the extent of the loss; in the present series of experiments there is an inverse relationship (see Table I). However, the variations in individual loss are found to be reduced if calculated per kg. body weight, or per square metre body surface. The relative constancy from individual to individual suggests that the loss is conditioned by the level of the endogenous metabolism; this possibility is substantiated by an examination of the more extensive data given for the endogenous N output, such as those

of Deuel *et al.* [1928], in which the value of 0·0241 g. per kg. body-weight was the daily excretion of urinary N after the subject had lived for more than a month on a protein-free diet—a figure which strikingly resembles the figures given in Table XI.

Is it by mere accident that there is a similarity between the daily amount of endogenous N excretion and the daily loss of N which occurs in these separation experiments, or does it really imply that the separate ingestion of carbohydrate and protein results in an inability of the organism to make good the endogenous loss of tissue protein? It must be remembered that only in Exp. 5 was the separation of carbohydrate and protein maintained over a prolonged period, but in this experiment the evidence is not clear-cut, for the separation was intermittent. We cannot therefore exclude the possibility that this loss of N and S might diminish in the course of prolonged separation, perhaps owing to the development of some compensatory mechanism. Exp. 5 does however clearly indicate that the N loss is accompanied by a decline in body-weight (Table VI). The significance of the other results of this experiment are discussed below.

The possibility of an endogenous source for the N lost is also supported by an examination of the separation of the 24 hr. urines into day and night fractions (Exps. 3 and 4). It is seen that the loss of N during the separation of the two foodstuffs is equally distributed between each fraction. This would apparently indicate a source of N which is unaffected by digestive and absorptive processes, i.e. an endogenous source. If this is so one might anticipate that the output of S would follow the same course, but examination shows that S loss is mainly confined to the day urine (Figs. 3 and 4). It is common knowledge that the metabolism of the S moiety of protein generally occurs more rapidly than that of the N fraction.

As regards the nature of the material lost during separation, Table XI shows that the N:S ratios of this material resemble closely the N:S ratio of muscle-protein (15·5:1), but it must be admitted that the quantities of N and S involved are small. (In Exp. 3, the S estimated was preformed sulphate-S, and accordingly the appropriate ratio is urea-N: preformed sulphate-S.) This finding of an N:S ratio similar to that of muscle is not however proof that the loss was from such a source; the material involved may well have been derived from a storage protein of similar ratio, utilized at the time of separation to replenish the endogenous requirements. It is significant that the creatinine excretion was relatively unaffected during the course of these separation procedures.

II. Exp. 6 indicates that for the normal metabolism of the dietary protein only a small fraction of it requires to be ingested simultaneously with the carbohydrate. In this experiment protein corresponding to only 4·64 g. N out of the 18·8 g. N in the diet was taken with the dietary carbohydrate, and yet the N output was the same as when all the protein (18·8 g. N) was taken with the carbohydrate (Table VIII).

The corollary does not appear to hold, viz. that the association of a small amount of carbohydrate with the protein of the day will necessarily maintain normal protein metabolism and prevent a loss of N (Exp. 5, Table VI).

III. By way of contrast to these N-sparing effects of carbohydrate in an adequate diet, the ingestion of carbohydrate in excess of energy requirements produces an actual storage of N. This is shown mainly in the day fraction of the 24 hr. urine, suggesting that it is the immediate products of digestion which are affected by the surfeit. The excretion of S is similarly depressed, the fall being again confined to the day output (Fig. 5).

It was observed by Larson & Chaikoff [1937], in their one-day superimposition experiments on dogs, that surfeit lost its N-storing effect when applied more than 4 hr. before or after the protein meal. With this we are in agreement, but we would also add that over longer periods than a single day, surfeit carbohydrate ingested separately from protein does eventually exercise a saving effect similar to that of surfeit carbohydrate taken simultaneously with the protein. The distribution of the saved N between the day and night fractions of the 24 hr. urines is identical under both conditions, viz. when the carbohydrate is taken apart from the protein (in the late evening) or along with the protein (in the afternoon) (Fig. 5).

IV. The possibility that the phenomena observed in Exps. 1 and 2 might be due to the change in the number of the protein-containing meals in the course of these experiments is discounted by Exps. 3, 4 and 5, to which this objection does not apply (Figs. 3, 4 and Table VI). It is also negatived by Exp. 8, in which the number of meals of a fixed diet were varied from 8 to 2 without seriously affecting the level of N output (Table X). This is consistent with the results of other workers, as reviewed by Cathcart [1921]. It may be further noted that this change in the number of meals in Exp. 8 did not alter the utilization of the protein and fat, since both faecal N and faecal fat were found to be unaltered in amount by this procedure. It would indeed appear that no appreciable economy in food is effected by eating more frequently than twice a day.

Mechanism of N-sparing and N-saving

In the foregoing discussion we have presented two separate aspects of carbohydrate in its relation to protein metabolism. These two may be termed N-sparing in the case of the adequate diet (Exps. 1–5), and N-saving in the case of the surfeit diet (Exp. 7). They differ in certain respects.

The separate ingestion of the carbohydrate and the protein of the adequate diet results in a loss of N from the body, and this loss may be spared if the carbohydrate and the protein are ingested together. To produce this sparing effect requires the close approximation of the protein to the carbohydrate (Figs. 1, 2, 3, 4 and Table VI). It occurs at once on ingesting the carbohydrate along with the protein; moreover, the sparing is equally distributed over the day and night excretions of N (Figs. 3 and 4). The N spared is not necessarily proportional to the amount of carbohydrate present along with the protein (Table VI).

The N-saving effect of carbohydrate present in excess of energy requirements differs from the N-sparing effect in that surfeit carbohydrate ingested at any period of the day is capable in the long run of causing this storage of N in the body (Fig. 5), in that it takes several days of surfeit to exert its full effect [Cuthbertson & Munro, 1937] and in that, in contrast to N-sparing, N-saving is mainly effected on the day excretion of N, even if the carbohydrate be taken at night (Fig. 5). The N thus stored in the body is roughly proportional to the amount of surfeit carbohydrate taken.

In seeking to explain the loss of N from the body which results from the separate ingestion of the protein and the carbohydrate in an adequate diet, four hypotheses may be considered. All four assume that the underlying factor is an increased immediate disposal of the food protein, leading to an insufficiency of protein remaining to replace the endogenous N loss.

(*a*) If an insufficient quantity of energy were present in the protein-fat meals of the separation period, it might make necessary an excessive utilization of food protein for immediate energy purposes; hence, less protein would be left over for tissue needs.

(*b*) The absence of carbohydrate from the protein-fat meals might have to be made good by production of carbohydrate from food protein, thus lessening the amount of protein available for endogenous requirements.

(*c*) In the absence of carbohydrate from the protein-fat meals, a more rapid combustion of protein by its own specific dynamic action might occur.

(*d*) A specific inhibitory action of carbohydrate on the deaminases of the body might exist, an inhibition which would not come into play unless both carbohydrate and protein were simultaneously ingested. This last theory can be made to embrace both N-sparing and N-saving effects.

The first of these hypotheses may be disposed of by reference to Tables II–V, in which it is shown that there is no deficiency of energy in the protein-fat meals of the periods in which carbohydrate and protein were separately ingested. One cannot therefore ascribe the N loss in these experiments to insufficiency at any particular period of the day, unless it be assumed that fat is not metabolized at a sufficiently rapid rate to cover the energy needs immediately after the protein-fat meals of the day.

The second argument seeks to explain the N loss as an attempt to make available the non-N residues of protein metabolism for carbohydrate synthesis. It is true that these residues would tend to compensate for the absence of carbohydrate in the protein-fat meals of the separated diet, but they would also be productive of an excessive deamination of the protein of the diet and a consequent protein-starvation of the tissues. This suggestion would seem unlikely in the face of the adequate carbohydrate available at other periods of the day (Tables II–V). It might of course be further contended that for the complete oxidation of the fat present in the protein-fat meals, newly formed carbohydrate might be required, thereby increasing the demand on the non-N protein moiety. If such be the case, the large variations in the quantity of fat present in the protein meals of our diets might be expected to cause proportionately varying losses of N. Such was not the case, the losses of N being on the contrary reasonably uniform (Table XI).

The third consideration is that, in the absence of freshly ingested carbohydrate, the specific dynamic action of the dietary protein must be met at the expense of the protein itself. That this is unlikely is suggested by a study of the dietaries of Exps. 1–4 (Tables II–V). In the period of separation of protein from carbohydrate in Exps. 1 and 2, the 2 meals of protein were separated from those containing carbohydrate by some 4 hr., whereas in Exps. 3 and 4, the periods of separation of the first protein meal from the first carbohydrate meal were 7 hr. and $8\frac{1}{2}$ hr. respectively. Since specific dynamic action after a protein meal is a prolonged effect [Williams *et al.* 1912], one would expect a commensurately greater loss of N in the second pair of experiments, to say nothing of variations dependent on protein intake, if such losses were actuated by specific dynamic action. Such a view is not tenable in the light of the relative uniformity of our results (Table XI).

We prefer to believe that both N-sparing and N-saving have one underlying mechanism, suggested by the observation of Krebs [1935] that deamination by liver slices is inhibited in the presence of easily oxidizable substances such as lactate, pyruvate, succinic and α-keto acids (but curiously enough, not glucose). In regard to the N-sparing of the ordinary adequate diet, the presence of carbohydrate (perhaps yielding a more active form of glucose within the body) along with the protein is sufficient to secure that some of the amino-acids escape deamination and go to replace the effete products of endogenous metabolism; N balance is thus attained. However, if all the dietary protein be ingested

separately from the carbohydrate, the deaminases are given a free hand, this sparing effect of carbohydrate no longer obtains and deamination is then complete. Further, to this waste of food N is added the unreplaced endogenous quota, so that there is produced an output of N which exceeds intake by an amount equal to the daily endogenous N.

The N-saving effect of surfeit carbohydrate may possibly be explained by the same mechanism; here, even more amino-acids are spared deamination by the inhibiting action of the greater concentrations of carbohydrate resultant on the surfeit intake. Thus in the blood a plethora of amino-acids is produced, and by the law of mass action the storage depots are further charged with protein. This would account for the finding of Larson & Chaikoff [1937] that, in one-day superimposition experiments, carbohydrate was only effective when ingested within 4 hr. before or after the protein, i.e. when its absorption fell within the main phase of protein metabolism. In more prolonged surfeit feeding (Exp. 7) storage of the carbohydrate would also seem to play a part, for surfeit taken outside the 4 hr. limits does have a N-saving effect in the course of a few days (Fig. 5), which probably represents the (variable) time taken to fill up the carbohydrate stores. The eventual result of charging these stores would be that the freshly ingested carbohydrate of the ordinary daily meals is unable to pass out of circulation with its wonted rapidity; it thus produces a higher concentration of carbohydrate within the organism than it normally would and simulates thereby the effect of giving a large draught of surfeit carbohydrate along with the meal. This would of necessity imply that owing to the prolonged period of surfeit feeding the Staub-Traugott phenomenon would be less marked, indicating a less effective disposal of freshly absorbed carbohydrate. Whatever may be the mechanism involved, carbohydrate ingested apart from protein seems to produce an inhibition of deamination of exactly similar extent and time-distribution to that of surfeit carbohydrate taken along with the protein.

That this egress of the carbohydrate from currency and that the availability of stores for this purpose play an integral part in determining the effect of dietary carbohydrate on protein metabolism seems more certain from the results obtained in Exp. 6. Here 2 meals of an adequate (but not surfeit) diet were loaded with all the dietary carbohydrate (some 390 g.) but yet a similar N balance was obtained as when there was a distribution of the same carbohydrate over all 4 meals of the day. That is, if there is excessive ingestion of carbohydrate at one particular meal at the expense of carbohydrate at other meals of that day, the excessive ingestion is at once counteracted by a legacy (from the carbohydrate-poor meals) of stores depleted in carbohydrate. The fluctuations in concentration of current carbohydrate at the sites of deamination would thus be more or less similar after each meal, even if the dietary carbohydrate were disposed unevenly between the different meals. We are of the belief that such a mechanism is the basis whereby N balance is made possible in the organism on the ordinary adequate diet, subjected as it is to vagaries of time and tide of carbohydrate intake.

This capacity for storage of carbohydrate probably also explains the curious results of Exp. 5 (Table VI), in which varying amounts of carbohydrate were given along with the dietary protein, in an attempt to reduce the loss of N which occurs on separation of carbohydrate from protein in an adequate diet. It was found that 100 g. carbohydrate so given were no more effective than 25 g., the N loss being equally reduced, but not abolished. Only when a larger proportion of the total dietary carbohydrate was transferred to the protein meals did N loss eventually cease to occur. This would seem to indicate that the carbohydrate stores at the times of the protein meals were not replete, but rather depleted, and

hence that 25 g. sugar and 100 g. sugar were equally well disposed of, the larger amounts being however less rapidly dealt with by the carbohydrate stores.

Further evidence of a carbohydrate mechanism in protein metabolism may be adduced from the experiments of Silver [1937] which indicate that the amount of carbohydrate in a diet is a factor in the establishment of N equilibrium on that diet. It was found that, by keeping the protein and energy content similar, but varying the carbohydrate-fat ratios of a series of diets, the N balance was favourably influenced by larger proportions of carbohydrate in the diet. The metabolism of the Esquimaux, and of other races whose dietaries contain very little carbohydrate, must presumably be such that the non-N moieties of the large quantities of protein in these diets are deviated to the filling up of the carbohydrate stores and thus successfully take the place of carbohydrate. In this connexion it is recalled that Voit [1881] showed that N and C equilibrium could be eventually established in dogs fed on a high meat diet in which virtually no carbohydrate was present.

Summary

1. It has been demonstrated on 4 human subjects that when the protein and the carbohydrate moieties of an adequate diet are separately ingested over short periods of time, there is a negative N balance of some 2 g. daily (mainly urea).

2. This loss is equally distributed between the day and night portions of the 24-hourly urines. There is a corresponding loss of S which, however, is confined to the day urines. Creatinine excretion is relatively unaffected by this procedure.

3. N metabolism is undisturbed by dissociating a part only of the dietary protein from the carbohydrate; this is in contradistinction to the N loss resultant on complete separation of protein and carbohydrate. The association of a small fraction of the dietary protein with the carbohydrate is sufficient to maintain N equilibrium.

4. Carbohydrate, if ingested over a sufficiently long period in excess of the energy requirements, causes storage of N and S no matter whether the carbohydrate be taken along with, or apart from the dietary protein.

5. The variation of the number of meals in a fixed diet from 8 to 2 causes a transitory loss of N due to the disturbance in time relationships. The digestibility and absorbability of the protein and fat of the diet are apparently unaffected by this procedure.

6. The changes in protein metabolism involved in these experiments and the possible mechanisms of their production are discussed.

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